
Engineering Microorganisms for Energy Production

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1 EXECUTIVE SUMMARY

JASON was asked by the Office of Biological and Environmental Research of the Department of Energy to assess the possibilities for using microorganisms to produce fuels as a metabolic product, in particular hydrogen or ethanol. We were asked to consider the prospects for achieving such biogenic fuel production in principle and in practice; and what the requirements and fundamental limitations are for achieving viability.

General Findings Concerning Biofuel Production

Biofuels are advantageous because they inherently solve the storage problem posed by the diurnal fluctuation of sunlight. Additionally they make carbon-carbon bonds, which are high-value mobility fuels. Biofuels represent a significant opportunity to address energy issues. For example, Brazil has a successful ethanol market at the present time, and ethanol is an important transportation fuel in Brazil. There is however a gap between what biofuels can currently do and what we need them to do to become a viable material component to global energy (energy friendly, carbon neutral, and economic.) On the other hand, the science underlying biofuels is still in an early stage of development and much likely remains to be discovered and understood. There is probably room for significant improvement.

The efficiency of biofuel production is ultimately limited by the efficiency of photosynthesis for converting sunlight into fuels. Photosynthesis has an upper bound on its efficiency of $\approx 10\%$, of the total medium energy in sunlight into stored chemical energy based on the conversion efficiency of the primary photosynthetic proteins. On the other hand, the time-averaged primary productivity for C4 plants in the field is approximately 0.25% in the

best cases. There is thus a fortyfold decrease between principle and practice. Photosynthetic efficiencies are far below that of man-made solar devices, and are likely to remain this way for a long time. However, systems and material costs for photosynthesis are much less expensive so biofuel can possibly form an economically attractive energy production option.

Plants are not necessarily optimized to be energy conversion machines. For example food crops have been genetically improved to increase food production, and the efficiency of food production has not yet plateaued.

However, the reengineering of plants to improve biomass energy yield is a multi-axis problem, and will likely require more than single (e.g., genetic) modifications of single proteins. The photosynthetic machinery has evolved to optimize fitness in a complex environment: Biological systems are intrinsically complicated because of the multiple feedback and control loops that must be present to guarantee robust survival. As a result, modifying any one property will likely have a limited leverage. In attempting to improve the system, it is important to think about the whole system (organism, environment, product, process). Progress bridging these gaps requires a dedicated commitment to breeding and/or molecular and systems level analysis. These two approaches should be synergistic.

General Findings concerning Microorganisms for Biofuel production

Microorganisms present a great opportunity for energy science, and hence are a natural focus for the Department of Energy. Microorganisms are simpler than plants; they have smaller genomes and proteomes, and are easier to manipulate and culture. The enormous biodiversity of microorganisms presents a broad palette of starting points for engineering. Microorganisms

already make many metabolic products, some of which are useful fuels. It is likely that microorganisms will soon be synthesized *ab initio*.

The upper bound on efficiency for oxygenetic photosynthesis in microorganisms is the same as that for plants ($\sim 10\%$), based on the primary photosynthetic proteins. Experimental measurements of efficiencies of fuel production must account for all system inputs and losses, including (but not limited to) pumping and sweeping out of products, stationary state relative to standard state, and the light intensity dependence of product yield. Current microorganisms are likely not optimized for energy production of useful fuels. For example, hydrogen production from algae is arguably operating at present, at 0.05% efficiency.

Recommendations and Conclusions

1. Boosting the efficiency of fuel formation from microorganisms is an important research challenge for the twenty first century. It is perhaps **the** major technological application for the emerging field of synthetic biology. In addition to the exciting opportunities for producing ethanol or hydrogen, microorganisms, either individually or in communities, might be used to directly produce liquid hydrocarbons. Realizing this potential requires both fundamental and applied research, and is a natural focus for the Department of Energy.
2. Engineering fuel production from microorganisms is a *systems* problem, requiring manipulation of multiple feedback and control loops. Fuel production is strongly coupled to the photosynthetic machinery and vice versa. Progress in both creating products and improving product yield requires recognition of the systems nature of this problem.
3. The systems biology of microorganisms is more tractable than that of

plants, and thus microorganisms represent an excellent opportunity. The synergy between research into biofuel production by microorganisms and the Genomes to Life program is important and should be fully exploited.

4. Photosynthesis is an active and exciting area of current research, with major discoveries concerning the regulation and relative importance of components happening each year[6, 27]. These discoveries will play an important role in reengineering fuel production pathways in microorganisms.
5. The commonly quoted 10% upper bound in photosynthetic efficiency assumes that no energy is wasted in storing the photogenerated charges in chemical bonds. Additional losses will come from regulatory processes as well as maintenance energy expended to repair the components and insure system robustness. Until there is a systems level understanding of photosynthesis, it will be impossible to meaningfully bound the potential efficiency of photosynthetic fuel production.
6. Successful metabolic engineering requires a basic understanding of the system to be engineered. More understanding of photosynthetic regulation is necessary before metabolic engineering can reach its potential.
7. There is a pressing need for strategies to minimize the oxygen sensitivity of fuel-forming catalysts in biological systems. Hydrogenases, nitrogenases, and rubisco in C3 plants are all oxygen sensitive. Indeed, C4 plants are more efficient because they developed an independent mechanism to isolate the rubisco from oxygen. Photodamage is a key concern to any photosynthetic microorganism, and repair mechanisms have evolved to deal with this. Any new catalyst must be compati-

ble with the existing repair machinery, or that repair machinery must also be redesigned. Directed evolution might prove to be a particularly promising strategy for improving these properties.

8. There is some opportunity to reengineer the photosynthetic components themselves to yield even higher energy conversion efficiency to the primary charge-separated products. This is a grand challenge, because of the interconnectivity and feedback loops already mentioned.
9. For carbon-based fuel production, a significant improvement in photosynthetic efficiency could lead to CO₂ supply constraints.
10. Even with an optimistic assessment of the potential for improvements, photosynthetic efficiency will lag behind that of man-made technologies (e.g., photovoltaic solar cells). For engineered microorganisms to succeed in the marketplace, their systems costs need to be significantly lower; however we are not aware of any systems-based cost analysis for solar H₂ generation from microorganisms. Such an analysis is needed to definitively understand the likely viability of this technology.

2 INTRODUCTION

This study was commissioned by the Department of Energy, to consider the problem of designing microorganisms for energy production, with the goal of producing fuels (particularly hydrogen or ethanol) as metabolic products. JASON was asked to consider in particular two main strategies: the conversion of cellulose to monosaccharides and then to ethanol; and the coupling of photosynthesis with hydrogenase enzymes to produce molecular hydrogen. This report will assess the fundamental requirements and the technical barriers that need to be overcome for these to become viable technologies. We will also comment more generally on the fact that some of the core issues and constraints in improving microorganisms arise in biofuels more generally. Our analysis and conclusions will emphasize the overall importance of using microorganisms for producing energy: this is an important problem in basic energy science, whose solution will require synergistic interactions with genomics, synthetic and systems biology.

We were fortunate to receive briefings from a number of leading scientists in the community, listed in Table 1. We are most grateful for their willingness to contribute to this study and to discuss followup questions and issues with us.

Speaker	Affiliation
Eli Greenbaum	Oak Ridge National Laboratory
Ping-Chen Maness	National Renewable Energy Laboratory
Maria Ghirardi	National Renewable Energy Laboratory
James Lee	Oak Ridge National Laboratory
Charles Wyman	Dartmouth

In addition, the following people were consulted by email and provided additional resources for our program:

Chris Somerville, Stanford University

Krishna K. Niyogi, University of California, Berkeley

The organization of this report is as follows. In the next section we discuss the general context of the study and the critical role that a single parameter, the efficiency of converting incident solar energy into useable energy, plays in determining the viability of the technology. For comparison purposes, Section 3 summarizes competing technologies for converting solar energy to fuel. Section 4 provides a general discussion of photosynthesis and photosynthetic constraints, and lays out the major issues for improving efficiency. Section 5 provides a detailed discussion of the constraints on photosynthesis: the physical constraints, the constraints on biological components and the system level constraints. Section 6 provides a more detailed discussion of current work in engineering microorganisms for hydrogen production. Section 7 discusses methods that might be appropriate for bringing about improvements to photosynthetic energy production: in particular we discuss both metabolic engineering and directed evolution. Section 8 discusses our findings, conclusions and recommendations.

A recurrent theme in the report is that progress in surmounting the formidable (but possibly solvable) technical hurdles will likely require a more complete systems view of the regulation and control of photosynthesis than we currently have. Although there is a natural tendency to view photosynthesis and photosynthetic engineering from the standpoint of individual components (e.g., designing better enzymes for catalyzing critical reactions), the intricate control and feedback loops present in photosynthetic energy production require viewing this problem from the standpoint of systems biology.

Indeed, it is our feeling that from the point of view of technology development, this problem should be thought of and presented as **the** primary challenge for systems and synthetic biology in the coming decades.

3 GENERAL CONTEXT

The reason for this study is clear. The United States and the world is faced on one hand with increased energy demand, and on the other hand concern over the security of energy supply. Current annual energy usage in the U.S. is around 3.5 Terawatts, and the world energy consumption is increasing rapidly especially with the modernization of developing world countries. On the other hand there is serious concern about carbon dioxide emissions. The current level of 380 ppm compares with the preindustrial values of 280 ppm. Annual increases are currently at the level of 1.8 ppm/year—so that by the end of the century, if current levels were maintained and action is not taken, the CO₂ level will be 550 ppm, whereas even higher levels will be produced if fossil fuel consumption increases.

The only way of addressing this issue is to find a domestic (global) energy supply which can cope with demand, while limiting CO₂ emissions. There are, unfortunately, very few possibilities. Perhaps the only viable option for producing transportation fuels (as opposed to stationary energy sources) is energy production from photosynthetically derived products. These are carbon neutral in that the CO₂ burned from any carbon-based fuel generated from photosynthetic products is exactly that taken up by the plants to grow in the first place. A principal advantage of photosynthetically derived biofuels relative to traditional (man-made) solar electricity systems is that biofuels inherently solve the storage problem posed by the diurnal fluctuation of sunlight: they make carbon-carbon bonds, which are themselves high-value mobility fuels.

Before examining the specifics of what would be required for biofuels to make a significant impact on the U.S. energy market, it is worth remarking

upfront that although progress is required for real economic viability, biofuels represent a very real opportunity, not a fantasy. This is perhaps best demonstrated by the example of Brazil, which has long converted part of their sugar cane crop to ethanol. The Brazilian National Alcohol program was established in 1975 following the oil crisis of 1973; by 1996, Brazil created the energy equivalent of 136,000 barrels of petroleum by converting sugar cane to ethanol [16, 17]¹.

How much energy can one expect from biofuels? The time-averaged irradiance in mid-latitudes, averaged over the year, day/night and weather patterns, is $200\text{W}/\text{m}^2$ (See Figure 1).

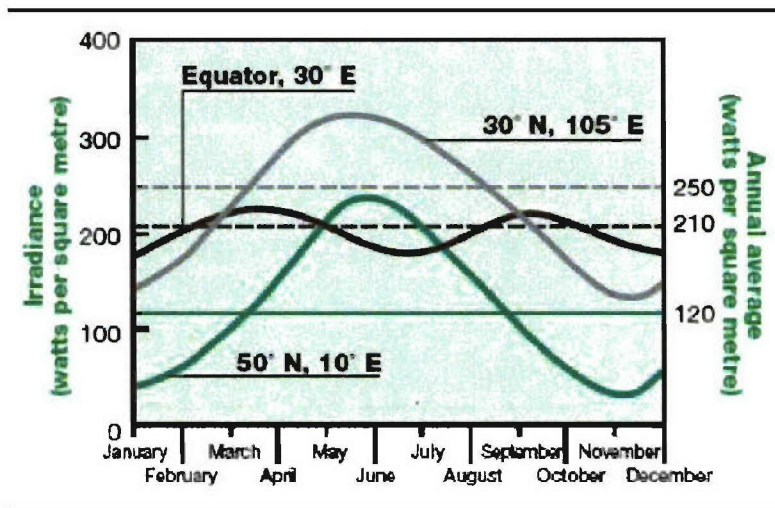


Figure 1: Irradiance from the sun, measured in W/m^2 . World energy assessment.

For energy from biomass to be a viable CO_2 neutral energy supply, it is necessary that this energy source is able to replace a substantial fraction of the current U.S. energy usage. We can therefore ask how much land area is

¹This compares with US petroleum consumption of 20 Million Barrels/day.

needed to produce 1 Terawatt of energy. This area is given by the equation

$$200 \frac{\text{W}}{\text{m}^2} \times \text{Efficiency} \times \text{LandArea} = 1 \text{ TW}. \quad (3-1)$$

Hence, the land area depends inversely on the efficiency for the conversion from the incident energy to fuel. The land area of current (agricultural) crop land in the United States is $\approx 1.8 \times 10^{12} \text{m}^2$. This implies that the number of land areas the size of current U.S. crop land $\#_{\text{crop}}$ that is needed to produce a Terawatt of energy is given by

$$\#_{\text{crop}} = \frac{0.28}{\text{Efficiency}[\%]}, \quad (3-2)$$

where the efficiency is measured in percent. Hence a conversion process that is one percent efficient requires 28% of current crop land, while a process that is ten percent efficient requires 2.8%. To help set a scale for these numbers, amorphous silicon solar cells currently operate at an efficiency of 5%, whereas crystalline silicon works at 15%. The latter is however extremely expensive, running at about $300\$/\text{m}^2$, or $10^{13}\$$ per Terawatt of energy, very far from being economical relative to current fossil energy for primary energy production.

Substance	Energy Density(MJ/kg)
H_2	141.8
Crude Oil	44
Coal	30-40
Sugar cane	16
Wood	16
ethanol	28

How well do current plants do? The global net primary productivity (the storage of net biomass or carbon in plant matter) varies from about $0.5\text{kg}/\text{m}^2/\text{yr} - 2.5\text{kg}/\text{m}^2/\text{yr}$, with most of the U.S. giving about $1\text{kg}/\text{m}^2/\text{yr}$

(See Figure 2). If we assume an energy content of 16MJ/kg (corresponding to both sugarcane and wood, see table), we find an energy content of 0.5W/m^2 . The efficiency is therefore $\approx 0.5/200 = 0.25\%$. From Equation (3-2), to produce 1TW of energy, we therefore need a land area corresponding to approximately the entire current U.S. crop land. And this would be sufficient in practice only if there were no inefficiency of transforming biomass into useful fuel. In fact converting the biomass to ethanol using current technology results in cutting the efficiency roughly in half or more, thus at least doubling the required land area [36].

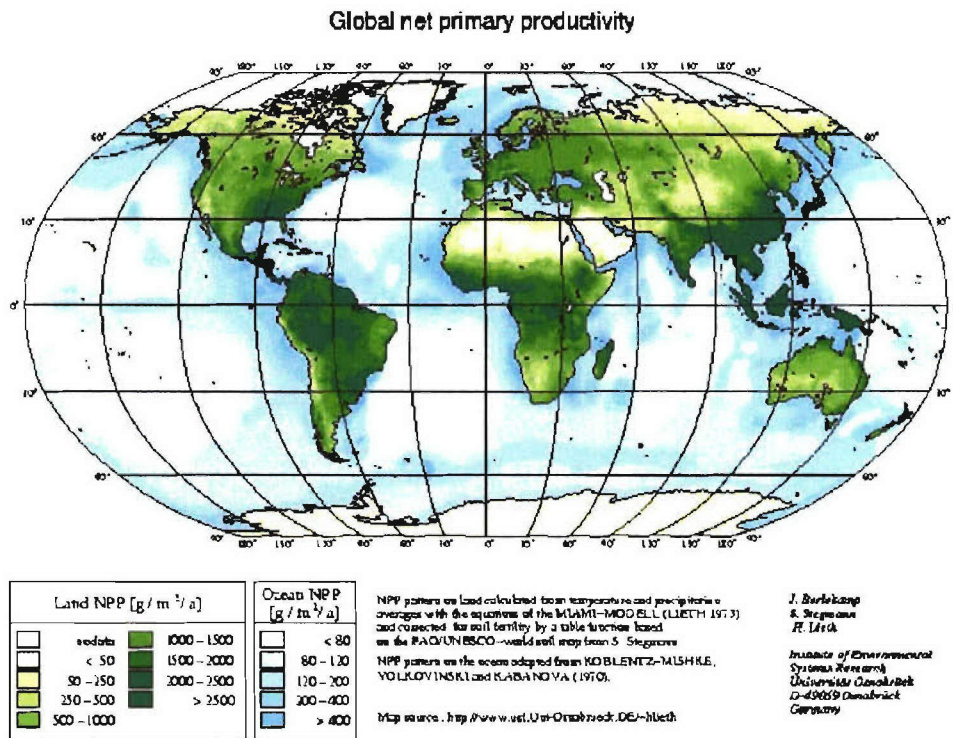


Figure 2: Global net primary productivity. (From Institute for environmental systems research, Osnabrück, Germany)

These simple arguments expose the two major hurdles we face for biomass energy production to be a significant contributor to U.S. or global energy:

1. The photosynthetic efficiency of converting incoming sunlight into biomass is sadly quite low. Even if there were no other losses, the land area required for biomass to be a competitive energy source is formidable.
2. The actual efficiency is further cut because the energy produced by photosynthesis is in the wrong form. For example, wood is not appropriate for powering cars.

It is also worth noting that light is not the only resource required for photosynthetic production of biomass: water, carbon dioxide, and other nutrients are also required.

4 COMPETING SOLAR TECHNOLOGIES

Before turning to our analysis of photosynthesis, it is worth summarizing the attributes of the technologies that are currently the most efficient for converting sunlight to fuels.

4.1 Photovoltaic Solar Cells

The highest efficiency route to fuel production from sunlight currently involves a photovoltaic (PV) cell connected in series to an electrolysis unit. In this approach, the photovoltaic cell is optimized for capture and conversion of sunlight into electrical energy. Single junction, flat-plate Si solar cells under 1 Sun illumination have conversion efficiencies of 15 – 20%. GaAs solar cells have conversion efficiencies of 20 – 25%. Multi-junction solar cells can have efficiencies of 30–32% under 1 Sun illumination, and higher efficiencies under concentrated sunlight.

Electrolyzers for formation of H_2 and O_2 from H_2O can have energy conversion efficiencies of in excess of 80% based on the electrical energy input divided into the energy of the fuels output from the electrolyzer. Hence, the overall system efficiency of fuel formation produced by connecting a photovoltaic cell with an electrolysis cell is, to a good approximation, 70 – 80% of the efficiency of the photovoltaic cell itself. Thus, overall system energy conversion efficiencies for fuel formation of in excess of 20% can be obtained by a PV array electrically connected to an electrolyzer unit.

This approach is the benchmark for energy conversion efficiency because the photovoltaic cell can have its band gap optimized to match the solar spectrum, either for the situation of a single band gap, for multiple

band gaps with multi-junction systems, and/or under concentration. Under concentrated sunlight, integration of the electrolyzer unit with the PV cell array and heat produced by the concentrated sunlight at the focal region can produce still higher system efficiencies, because some of the solar heat can be transferred to raise the temperature of the electrolyzer, reducing the voltage needed to split water (for an entropically favored reaction like $\text{H}_2\text{O} = \text{H}_2 + \frac{1}{2}\text{O}_2$, increasing the temperature favors formation of the products). Other systems that also have very high efficiency involve intimate integration of the electrolysis function with the photovoltaic cell, and do not involve a separate electrolyzer unit. Instead, the electrocatalysts for reduction of protons to hydrogen and for oxidation of water to oxygen are plated onto separate sides of a multijunction photovoltaic cell, with the cell designed to produce sufficient voltage at maximum power (typically 1.4-1.5 V) to sustain the electrolysis of water, instead of the production of electricity. Such systems have overall energy conversion efficiencies of 10 – 17%, depending on the exact details of the system.

4.2 Solar Thermal Systems

Another route to fuel formation from sunlight involves concentrated solar thermal systems. Under high optical concentrations, very high temperatures can be produced in the focal region of the optical path. Such systems require dual axis tracking of the sun, but replace relatively expensive photovoltaic cells with less expensive optics as the main areal component of the system. The use of heat instead of light absorption in a photovoltaic cell at the focal point is conceptually advantageous in the formation of fuel. The reason for this is that photon conversion devices all suffer efficiency losses due to their inability to absorb photons below the lowest band gap region in

the device, and photons of higher energy than the band gap in any region of the device generally thermalize rapidly and are only converted as if they had band gap excitation energy. These well-known (Shockley-Quessar) restrictions set a limit on the ultimate conversion efficiency of a photon conversion system. In contrast, a solar thermal system can be used to generate high temperatures in a reactor, which then in principle is limited in efficiency by the second law $(T_1 - T_2)/T_1$ term; with T_1 of 2000 K and T_2 of 300 K, efficiencies of $> 80\%$ are possible in principle for direct conversion of sunlight into chemical fuels.

The keys to making such systems work in practice involve: a) engineering design to allow for good optical paths and photon capture into the reactor, while minimizing re-radiation of absorbed, thermalized photons back to the environment; b) developing a set of closed-cycle chemical process steps that can make the desired fuels, and c) developing materials for the solar thermal chamber construction that are compatible with the high temperatures and reactants used in the thermochemical cycle. For water splitting, a two step process that looks especially promising involves $\text{Zn} + \text{H}_2\text{O} = \text{ZnO} + \text{H}_2$, and then $\text{ZnO} + \text{H}_2\text{O} = \text{Zn} + \text{O}_2$. The engineering issue is that the two process steps must be performed in batch mode and isolated temporally from each other. Additionally, the chamber materials must be compatible with the mass flows of reactants and products at the very high temperatures at which such systems need to operate to produce a rapid rate and a good conversion to products at each step. In principle, such cycles could offer 60 – 70% overall energy conversion efficiency, if they can be assembled successfully into a practical concentrated solar power system.

4.3 Direct Photolysis

A third approach is to use direct photolysis, either with a semiconductor as the photocatalyst or with organic or inorganic dye molecules as the photocatalyst for the water splitting process. Semiconductor photoelectrochemistry functions conceptually as a photovoltaic cell hooked in intimate series with an electrolyzer unit, except that there is no metallurgical junction between two solid phases in the photovoltaic unit to produce an electric field and effect charge separation. Instead, the junction is formed by virtue of the contact between the solid and the water-containing electrolyte. Semiconductors that can spontaneously split water with sunlight, and which are stable in sunlight, are relatively well-known, and generally involve metal oxides with high band gaps, such as SrTiO_3 , KTaO_3 , or SnO_2 . The large band gaps of such systems preclude obtaining high efficiencies for solar fuel formation, but there is sufficient solar photon flux in regions where they absorb that overall solar energy conversion efficiencies of c.a. 1%, i.e., greater than that of most plants, has been demonstrated. Generally, oxides and other semiconductors with smaller band gaps, that are better suited to capture photons from the solar spectrum, are not robust in water and either oxidize (like Si to SiO_2) or corrode (such as GaAs to Ga^{3+} and As^{3-}). Recently developed materials have expanded the light absorption into a portion of the visible region of the solar spectrum, and efficiencies for water splitting of approximately 4 – 5% have been reported. Work on semiconductor photoelectrolysis being performed currently emphasizes development of new materials or materials combinations that can combine stability with high efficiency for fuel formation in water, following on the 4 – 5% efficient materials that have been developed recently.

Each of the above approaches has its own tradeoff between cost and efficiency. More details on these various approaches can be found in the recent, comprehensive report by the DOE entitled Basic Research Needs for Solar Energy Utilization². A key differentiator between these various high efficiency methods of H₂ formation from sunlight and biomass is their balance of systems costs. Photovoltaic cells, which are highly efficient, are relatively costly per watt of electricity produced at peak power generation (dollars/W_p), due both to the cost of the cells, modules, and the wiring and needed electrical interconnections (for ac power also the cost of the inverter and grid connections must be considered). Concentrated solar power systems produce the current lowest cost methods for producing solar electricity, at between 10-15 cents/kW-hr fully amortized systems costs at electric utility scale installations. In such situations, land area costs are not the driving factor, and the cost/W_p of electricity is the key economic figure of concern.

4.4 Do Biological Systems Stand a Chance?

Given these very high efficiencies, do biological systems stand a chance? The hope is that the balance of systems costs will be significantly lower, to compensate for the strong likelihood that the overall energy conversion efficiencies to fuel formation will be significantly lower. The hope is that the cost to install a biological system that can grow and reproduce will be low, and that the cost of harvesting the fuel and collecting it back to the central station will be low as well. Other than conventional biomass, however, we know of no systems-based cost analysis for solar H₂ generation from, for example, large algae ponds, that would allow one to more precisely evaluate the degree to which the systems costs can in fact be lowered compared to those

²The lead author on that report is one of the members of this study.

of concentrated solar power systems or solar electricity systems connected either intimately or in series to electrolysis units. (A preliminary analysis was presented in [2]). Such is needed to evaluate whether the relatively low efficiencies of fuel formation from biological systems can be compensated for by an ultra-low balance of systems cost so that such approaches could be in principle economically advantaged relative to the other methods described above.

5 (REDESIGNING) PHOTOSYNTHESIS

It is important in considering the low photosynthetic efficiency to recognize that photosynthetic plants and microorganisms have not evolved to produce energy for us in the form that we want it—indeed it is the very real opportunity for bioengineering to substantially improve the situation that is the subject of the present report. The canonical wisdom is that plants and microorganisms are designed to maximize reproductive capacity: for plants this presumably requires optimizing seed production and dispersal, a very different requirement than biomass productivity. Damage control (against the production of excess free radicals) is also of paramount importance.

Later on in this report, we will discuss in some detail the physical and biological constraints on photosynthetic energy yield. We will see that the actual 0.25% efficiency is well below the physical constraints. It is also well below the 10% light processing constraint imposed by the primary proteins of photosynthesis (Photosystems I and II). On the other hand, we will argue that (i) for carbon based fuels CO_2 supply constraints are likely to keep the upper bound below 10%; and (ii) we believe that it is premature to conclude that the only biological constraint is associated with these primary proteins.

To flesh this second point out, we first give an analogy, in the form of a parable. Imagine that Benjamin Franklin reappeared today (300 years from his birth), and came upon a modern day computer. Although he does not know how the computer works, he marvels at what it can do. At some point, he realizes that the computer would be more useful if it would only compute faster; and for this reason he decides to take the computer apart to discover what determines and limits the speed. The initial dissection leads him to the discovery of a remarkable oscillator (the clock) at the heart of the

computer, which can switch very fast indeed. He experiments with different computers made by different companies at different times and finds that the clocks have different speeds. He notices that the number of operations that the computer carries out per unit time has a positive correlation with the speed of this oscillator. He therefore reasons that making the computer faster is just a matter of improving the clock—and designs a research program to improve the speed of this essential component.

On the other hand, in reality, we know that computing is not just about clock speed. The design of a computer requires understanding and coordinating a myriad of other issues including memory access time, bandwidth, software, hardware architecture, interconnects, etc. Indeed, increasing the clock speed too much can lead to instabilities in the processing of the computer. Improving the computer requires synergistic changes in the entire system—the modification of an individual component is in general not effective, or, in any case, has limited leverage.

The photosynthetic production of biomass or useable energy likewise requires coordination between the various active components—from the proteins converting light into electrons to the enzymes catalyzing the conversion of CO_2 to sugars. Tuning any individual component should therefore be expected to have limited leverage. Indeed, in a biological system, feedback loops are how evolution ensures robustness.

Despite the complexity of the problem, there are grounds for being optimistic that increased energy production can be achieved. A pertinent example is the case of food crops. Crop yields have increased (essentially linearly) over time to the present day, and have shown no sign of saturation. Improvements have come from combinations of nutrition, pesticides and breeding. One could imagine that a concerted effort at improving energy producing

organisms (crops or microorganisms) would also lead to substantial improvements over current yields.

6 PHOTOSYNTHESIS AND PHOTOSYNTHETIC CONSTRAINTS

Figure 3 shows a schematic block diagram of how photosynthesis works to convert light into useable products. First, light is absorbed by the photosynthetic machinery: this is a collection of proteins (described in some detail below) which absorb the light and then convert it into charge-separated chemical species (electrons and protons), which are subsequently converted into ATP, NADPH, and O_2 . The first two of these products then provide the energy to catalyze the reactions to create products in the cell. Such products can take a wide variety of forms: solids (such as cellulose, hemicellulose and

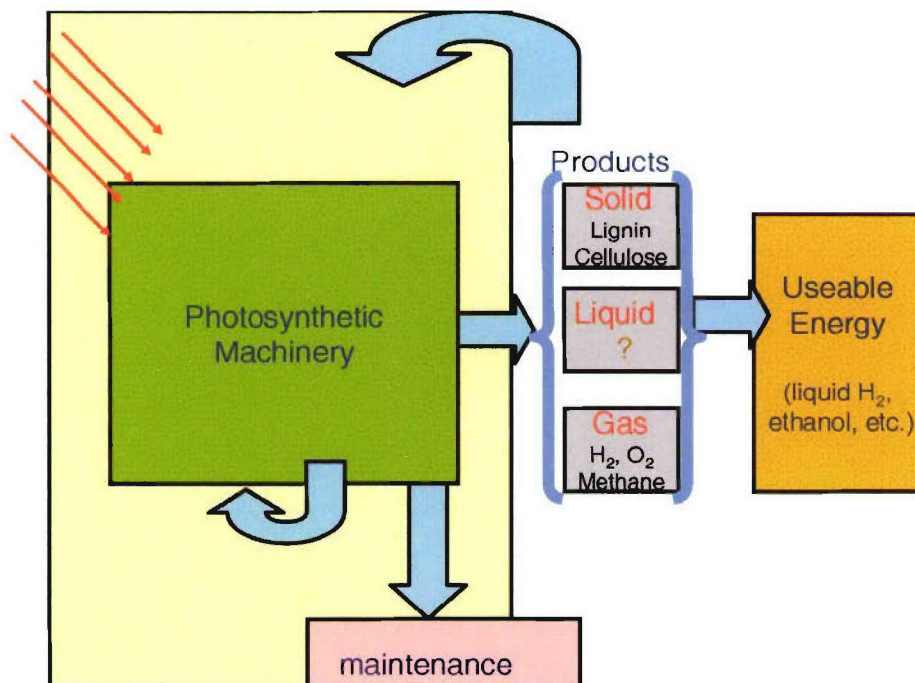


Figure 3: Block diagram of photosynthesis. Incoming sunlight is converted to electrons and protons (NADPH and ATP). These in turn are used to create photosynthetic products, and to maintain the organism.

lignin); gases (such as molecular hydrogen, or methane) or perhaps (with suitable reengineering of the system!) even liquid fuels, (i.e., hydrocarbons of ethanol.)

All of the photosynthetic energy converted to ATP and NADPH cannot be used for producing product: some fraction of it must be used in the maintenance of the system—both to regenerate the photosynthetic proteins when they become sufficiently damaged, and to maintain the photosynthetic organism itself. It is also important to note that *the pathway from light to reaction products is not open loop*. There is a negative feedback loop on the product stream that impacts how efficiently light is converted to ATP and NADPH. If, for example, in a photosynthetic organism they use the Calvin cycle to produce biomass, if too much ATP and NADPH is produced to be utilized by the Calvin cycle and/or metabolism, the production rate is attenuated. Likewise, the stoichiometry of ATP to NADPH is under tight control.

Finally, to be useful energy sources for society the direct products of photosynthesis must be converted to a form of useable energy. Examples of this include converting cellulose to ethanol for use in transportation applications.

6.1 The Photosynthetic Apparatus

How does the photosynthetic apparatus work? Photosynthesis (from the absorption of light to the production of fuels) in both plants and microorganisms occurs in the chloroplast, a membrane-lined cavity that is a few microns in diameter. Inside the chloroplast is a highly invaginated membrane, the

thylakoid membrane The core photosynthetic proteins are membrane proteins that are located on the thylakoid.

6.1.1 Light reactions

Figure 4 shows a schematic of the various proteins and transport mechanisms that take place. The two core photosynthetic proteins are called Photosystem I and Photosystem II (PSI and PSII). Each of these proteins has an antenna system of absorbing pigments that increases the rate of light absorption.

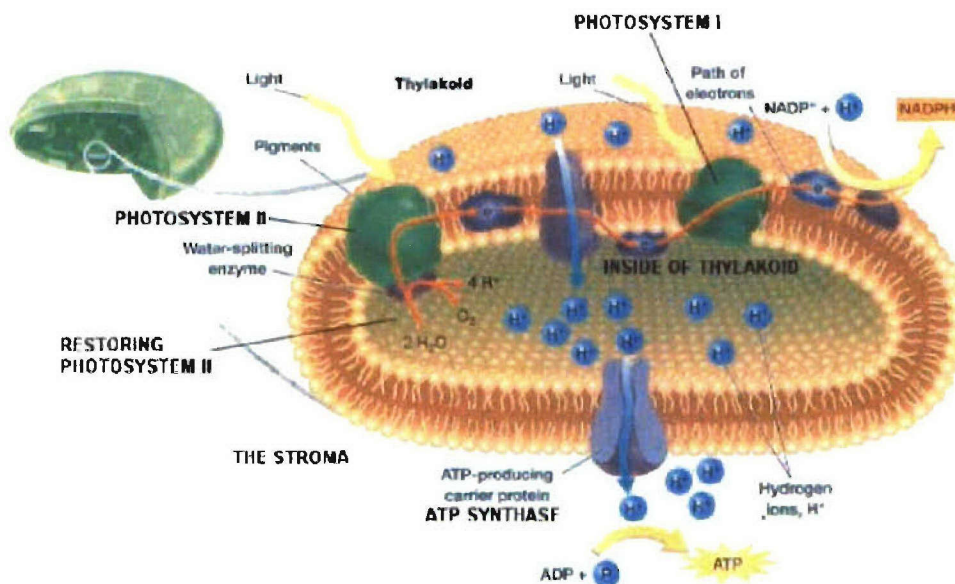


Figure 4: Sketch of the basic photosynthetic machinery. Taken from <http://www.sirinet.net/jgjohnso/Ithylakoidmem.jpg>

Light is first absorbed in PSII: this protein performs the water splitting reaction to evolve O_2 from H_2O , keeping the protons thus produced inside the thylakoid. After receiving excitation energy from the antenna system,

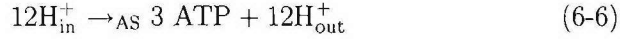
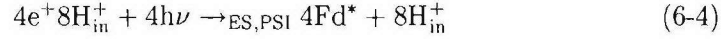
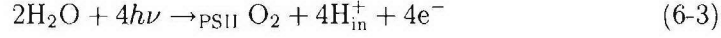
the primary charge separation process occurs in the photosynthetic reaction center. The reactions are highly efficient: at each point, the forward charge transfer reaction rates are about 100-500 times more rapid than the relevant back-reaction rates (i.e. than recombination to eliminate the electron-hole pair and produce only heat), so the quantum yield at each step, and overall, for the charge separation process is very close to 1.0. In this fashion, the solar flux absorbed by the antenna pigments is converted into a separated electron-hole pair that can be used to drive the chemical reactions in photosynthetic metabolism.

These electrons are shuttled from PSII to PSI by mobile electron carriers: plastoquinone (which diffuses in the membrane from the photosystem to the cytochrome complex, which binds the mobile electron complexes) and plastocyanin (which is soluble inside the thylakoid membrane, and moves electrons to PSI).

Photosystem I then converts the mobile electron into an intermediate energetic product. The resulting mobile electron is then transported across the thylakoid membrane to an electron acceptor, ferredoxin. Once across the thylakoid membrane, there are two possibilities: In the first mode of operation (dubbed “linear electron flow” (LEF)) the electron combines with NADP^+ to form NADPH. In the second mode of operation (dubbed “cyclic electron flow”) the electron can react with plastoquinone and thus cause protons and electrons to be transported back across the thylakoid membrane.

The pH gradient produced by both the water splitting reaction in PSII and the cyclic electron flow around PSI is used by the F_0F_1 ATPase to produce ATP outside the thylakoid membrane.

In total, the light reactions of photosynthesis are as follows:



The reaction (6-3) takes four photons to split two water molecules, catalyzed by PSII; the reaction (6-4) converts the electrons and protons to reduced ferredoxin Fd^* as well as protons inside the thylakoid membrane (H_{in}^+); reaction (6-5) converts Ferredoxin into NADPH, using the $\text{Fd} - \text{NADP}^+$ reductase (FNR); finally protons inside the thylakoid membrane are converted to protons outside the thylakoid membrane by the ATP synthase (AS), resulting in the production of ATP. Altogether, 8 photons split 2 water molecules to form 2 NADPH and 3 ATP, which can be used to fix one molecule of CO_2 (see Figure 5).

The energetics of these photosynthetic reactions are summarized in the famous Z-scheme: the water splitting reaction of PSII requires a photon of wavelength 680nm (1.8eV); the resulting electron is then transferred from the excited state of PSII to a quinone (resulting in an energy loss of about 0.8eV); the electron is then shuttled to PSI (resulting in an additional loss of about 0.4eV), where an additional photon of wavelength 700nm (1.8eV) is absorbed. The electron is finally shuttled to the terminal electron acceptor, ferredoxin—the energy loss here is about 1.2eV.

It is worth noting at this point one remarkable feature of the photosynthetic apparatus that is not apparent from looking at the Z-scheme. Namely, Photosystems I and II are spatially separated. Figure 6 shows a schematic of the spatial distribution of the proteins. PSI occurs primarily on the stacked

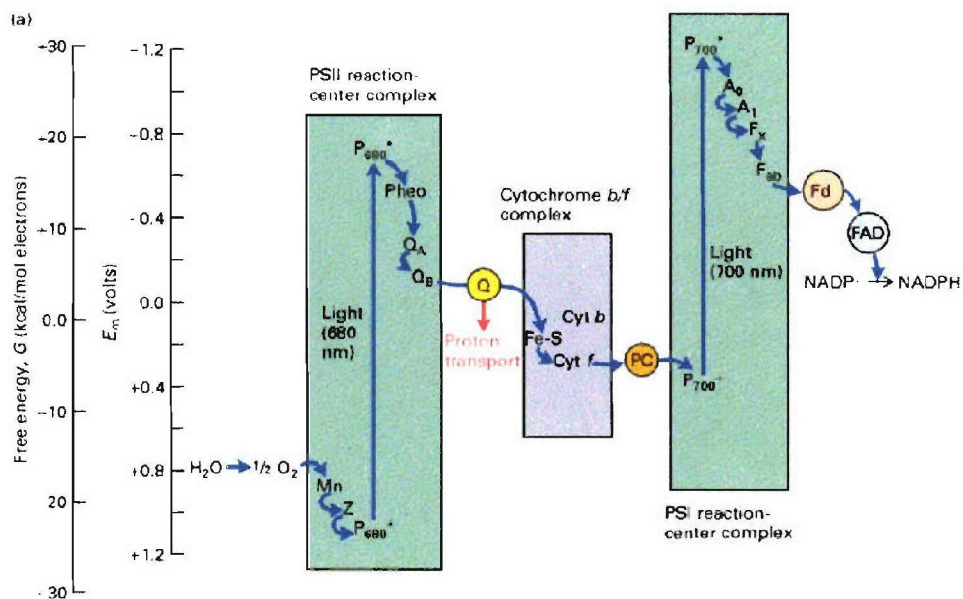


Figure 5: Energetics of photosynthetic proteins (from [22]).

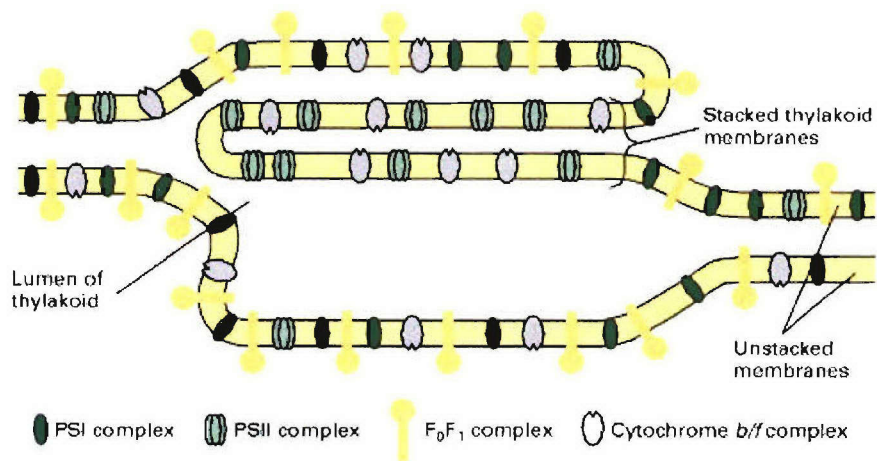


Figure 6: Distribution of Photosystem I and II in the thylakoid membrane (From [22]).

parts of the thylakoid membrane, whereas PSII occurs primarily on the unstacked parts. In particular, *the two protein complexes are not located right next to each other!* There is both an energetic and a kinetic cost for the spatial separation of the two protein complexes. From the Z scheme the energetic cost is about 0.4eV (about 10 percent of the total energy loss); from a kinetic standpoint the transfer of the electron from PSII to PSI requires diffusion through the interior of the thylakoid. If we assume a typical transport distance of about $1\mu\text{m}$ and a typical diffusion constant of a small molecule of about $10^{-5}\text{cm}^2/\text{sec}$, the time T_{diff} that it takes for a plastocyanin to diffuse from PSII to PSI is

$$T_{diff} \approx \frac{(10^{-4}\text{cm})^2}{10^{-5}\text{cm}^2/\text{sec}} = 10^{-3}\text{sec}.$$

This is the rate-limiting step for the overall reaction.

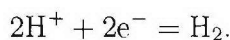
6.1.2 Dark reactions

The second part of photosynthesis involves reactions that convert the NADPH and ATP produced in the light reactions into final products. These reactions occur in the chloroplast outside of the thylakoid membrane. There are a myriad of different products that are produced by photosynthetic organisms. These include

1. CO₂ fixation. Here the reactions convert carbon dioxide to sugars, using the ATP and NADPH produced by the primary photosynthetic reactions. 3ATP and 2NADPH are required to fix a single molecule of CO₂; given the accounting of equations (6-3-6-6) fixation of a single CO₂ to sugars requires 8 photons. The reactions converting CO₂ to sugars are catalyzed by the enzyme rubisco (*ribulose 1,5-bisphosphate*

carboxylase). The reaction is very sensitive to pH, with greater efficiency at higher pH. Additionally, the reaction is highly oxygen sensitive: the enzyme rubisco presumably evolved when the atmospheric oxygen concentration was far below the present day value: when oxygen is present, approximately half of the carbon dioxide is lost to photorespiration. Much effort has been expended to reengineer rubisco to be less oxygen sensitive, to no avail [16].

2. H₂ production. Here one must catalyze the reaction



This reaction must be catalyzed by an enzyme. Two different possibilities are known in photosynthetic organisms. Cyanobacteria have nitrogenases which indirectly catalyze the production of H₂ (as well as NH₃). These reactions however require ATP: at least two molecules of ATP are required per electron. The other possibility is that some cyanobacteria and algae have a hydrogenase enzyme that directly catalyzes the reaction, without requiring any ATP. Green algae such as *Chlamydomonas reinhardtii* uses the [Fe]-hydrogenase which takes the electron from the reduced ferredoxin (Fd*)[15], the electron donor to NADPH in the photosynthetic pathway (Eq. 6-5). Cyanobacteria contains only the bi-directional [NiFe]-hydrogenase which takes the two electrons from one molecule of NAD(P)H to reduce the protons. The in vivo function of these hydrogenases are thought to be the electron dump needed for carbohydrate metabolism under anerobic conditions, when NAD(P)H accumulate due to the lack of the preferred electron dump, O₂. Most existing efforts on biohydrogen research have focused on the green algae *Chlamydomonas reinhardtii*, as cyanobacteria mainly consume nitrogen. Low rates of hydrogen evolution have been observed

for *Chlamydomonas* grown under anaerobic or sulfur-deprivation conditions.

The action of the hydrogenases is extremely oxygen sensitive: Exposure to atmospheric oxygen shuts the reaction off very quickly . There is also the additional problem that any electrons produced by photosynthesis could also be directed towards the Calvin Cycle (CO_2 fixation pathway); efficient hydrogen production requires figuring out how to direct only as much energy towards carbon fixation as is needed to maintain viability of the culture.

6.2 Physical Constraints

Now that we have described the photosynthetic machinery, we turn to a detailed discussion of what determines its efficiency, and how the efficiency could be improved. First we discuss the purely physical constraints on photosynthetic yield.

Photosynthetic reactions require the consumption of three resources: light, carbon dioxide and water. Having the required amount of each of these quantities available to the photosynthetic organism *at the location where the photosynthesis is occurring* gives physical constraints.

6.2.1 Solar spectrum

Figure 7 plots the solar spectrum, the intensity of light as a function of wavelength, and Figure 8 plots the absorption coefficients of the various photosynthetic pigments. The pigments span the range of the solar spectrum, and it seems to be a reasonable assumption that the pigments are distributed so as to effectively absorb all incoming photons.

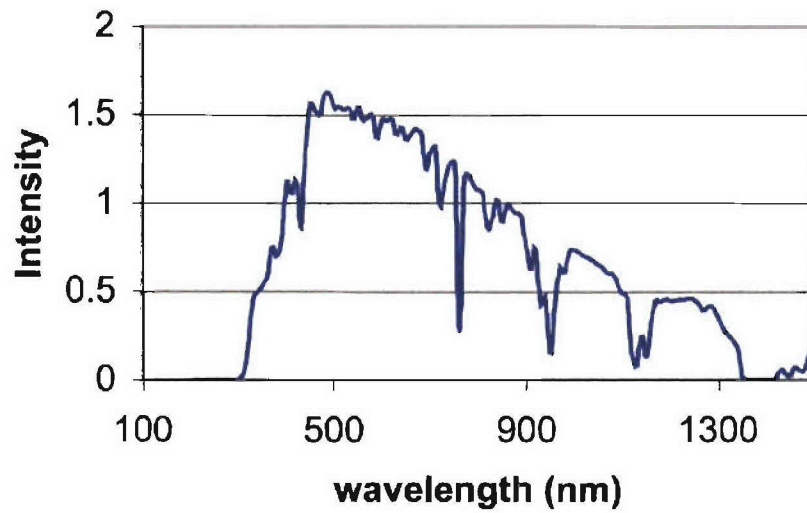


Figure 7: Solar spectrum: intensity of incoming solar radiation as a function of wavelength.

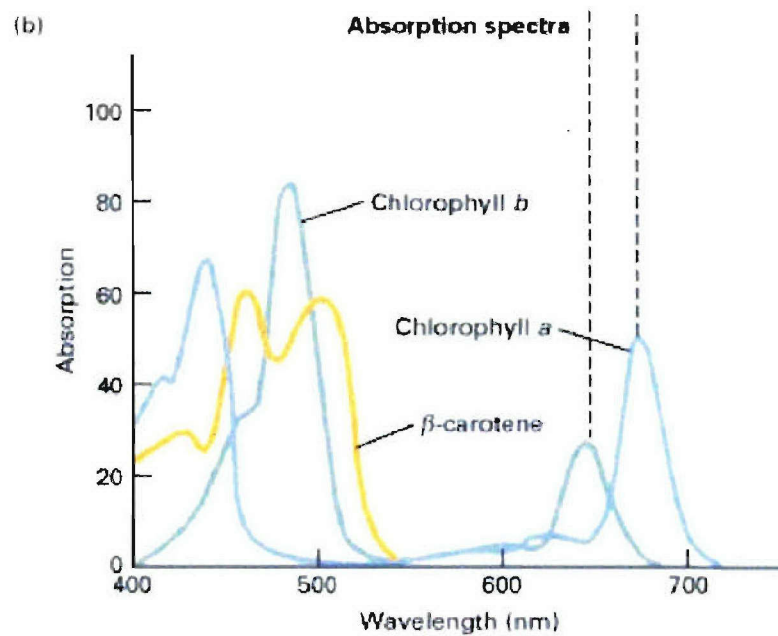


Figure 8: Absorption spectrum of photosynthetic pigment proteins (Lodish *et. al.*).

On the other hand, recall from the basic Z scheme of photosynthesis that the photosynthetic reaction centers require photons of wavelength 680nm and 700 nm. Absorption of photons with smaller wavelengths catalyzes the reactions, but results in a wasted energy corresponding to the difference between the photon energy and the energy needed for the reaction. If $N(E)$ is the number of incident photons in the solar spectrum between energies $E \rightarrow E + dE$, then the total energy in the solar spectrum is

$$E_{total} = \int_0^\infty N(E)E dE.$$

If $E^* = 1.8eV$ is the energy required for the photosynthetic reaction center, then the total energy processed by the protein is

$$E_{processed} = \int_{E^*}^\infty EN(E)dE.$$

The efficiency of this step is therefore

$$\eta_{light} = \frac{E_{processed}}{E_{total}} \approx 0.37,$$

where we have evaluated this number numerically.

The inefficiency of light capture is a physical constraint hard-wired by the solar spectrum, and it is difficult to understand how it could be improved, i.e., the framework of the known photosynthetic molecule machinery. Man-made solar cells get around this to some extent by using multiple band gaps.

6.2.2 Carbon dioxide flux

A second physical constraint comes from carbon dioxide flux. This constraint is of course only relevant when considering products such as ethanol, which result from the fixation of CO_2 . In particular, this constraint does not apply to hydrogen production.

The maximum CO₂ flux imposed by purely physical constraints is determined by transport processes in the atmosphere: what is the maximum flux of CO₂ that can be taken in by a photosynthetic absorber?

The flux is given by

$$D \frac{[\text{CO}_2]_{\text{atmosphere}} - [\text{CO}_2]_{\text{ground}}}{\ell} \quad (6-7)$$

where ℓ is the characteristic scale of the diffusive gradient, D is the atmospheric diffusivity, and $[\text{CO}_2]_{\text{atmosphere}}([\text{CO}_2]_{\text{ground}})$ is the concentration of carbon dioxide in the atmosphere and the ground, respectively. The maximum flux corresponds to the situation where the concentration of carbon dioxide at the ground is as small as possible, $[\text{CO}_2]_{\text{ground}} \approx 0$. Here, for simplicity, we are ignoring the partition coefficient of CO₂ within the leaf. The argument here assumes that the conductance of carbon dioxide to the leaf is limited by the diffusive boundary layer; for velocities much higher than 1m/sec it is in fact limited by the stomatal conductance [7].

What determines ℓ , the length scale of the vertical gradient? In the absence of an external wind, this is determined by the size L of the absorber. When a wind is present, the scale of the gradient is decreased, and (assuming laminar flow near the absorber) $\ell = L/\sqrt{UL/D}$. The assumption of laminar flow near the absorber is reasonable as long as the size of the absorber is smaller than the size of turbulent eddies in the atmosphere above.

If we assume a modest wind velocity $U = 1\text{m/sec}$, the absorber size $L = 1\text{cm}$ (the size of a typical leaf), $D = 0.15\text{cm}^2/\text{sec}$ and that $[\text{CO}_2]_{\text{atmosphere}} = 380\text{ppm}$, we find the theoretical maximum carbon dioxide flux is 5×10^{15} molecules/cm²/sec. This should be compared to the maximal observed assimilated carbon dioxide flux [11] of 2×10^{15} molecules/cm²/sec.

To give context to these numbers, what is the actual carbon diox-

ide consumed during photosynthesis? The solar flux corresponds to about 10^{17} photons/cm²/sec. where here we are discussing photosynthetically active photons (those with energy above 680nm). As discussed below about a factor of ten of these photons are absorbed by the light harvesting machinery and do not contribute to carbohydrate production. Additionally, as discussed above, CO₂ reduction consumes about 8 photons for fixing a single molecule of CO₂. Hence we find that carbon dioxide flux must be about 10^{15} molecules/cm²/sec to keep up with photosynthesis.

Interestingly, the required carbon dioxide flux is of order the maximum theoretical flux, for a 1 cm leaf. The numbers estimated above have order unity errors in the prefactors, and so we cannot conclude from this argument that CO₂ is the limiting factor for photosynthesis; on the other hand it seems evident that a several fold increase in photosynthetic efficiency (eliminating the photons absorbed that do not directly contribute to carbohydrate production) could run into a problem with carbon dioxide flux. Thus, it well may be that for fuel production involving carbon, photosynthesis is ultimately limited by a carbon dioxide constraint. Note that this constraint does not directly apply to hydrogen production.

How does this constraint apply to algae ponds? Here the length ℓ is set by the size of the pond. For sufficiently large ponds, the diffusion constant D is not the ambient diffusion constant, but is the much larger turbulent diffusivity of the atmosphere. A ballpark number for the atmospheric diffusivity is 10^4 – 10^6 cm²/sec [35], though the precise number depends on weather conditions. If the pond is one mile in diameter, the diffusive flux is $\sim 10^{15}$ – 10^{17} molecules/cm²/sec, larger than that estimated for a leaf (owing to the turbulent diffusivity in the air). For a 20-fold larger pond, the diffusive flux would be of order that in the air. The maximum for ℓ is set by the scale

height of the atmosphere –the characteristic scale over which the atmospheric diffusivity itself varies. This is of order $10km$, which leads to a CO_2 flux of 10^{14} – 10^{16} molecules/cm²/sec.

6.2.3 Water consumption

Photosynthesis produces one molecule of H_2O from four (photosynthetically active) photons. This implies we need about 2.5×10^{15} molecules/m²/sec = 4×10^{-9} Moles/m²/sec of water, or $\sim 10^{-10}$ Liters/m²/sec. For an area the size of the U.S. cropland ($\sim 2 \times 10^{12}$ m²) this is about 200 L/sec of rain, far below average annual rainfall. Hence yearly averaged water consumption is not (in principle) a problem—though of course fluctuations in rainfall of course presents challenges, they are well known to farmers.

6.3 Biological Constraints

6.3.1 Photosystem I and II

The basic requirements on the photosystems is that they on one hand have high quantum yield, so the backreactions are insignificant. Both PSI and PSII have forward charge transfer reaction rates about 100-500 times more rapid than the relevant back-reaction rates. On the other hand, in achieving this quantum yield there is a substantial energy sacrifice involved in the multi-step process converting photon to electrons. This energy cost occurs because of the simultaneous requirement of keeping the electron-hole pairs separated for a long enough time that they can be shuttled away from the reaction centers (where recombination can otherwise occur).

Compared to the charge carrier mobility in inorganic semiconductors, the mobility in photosynthetic pigments is relatively low and hence the electric field strength, and electric potential gradient, needed to spatially move the charge from pigment to pigment is relatively large. The Z scheme (Figure 5) of photosynthesis indicates that the energy of the initial excited state is approximately 1.83 eV, but the energy losses in transferring the charge from pigment to pigment are substantial. The final energy of an electron entering PSI from PSII is only about 0.6 eV. This leads to the efficiency of converting photon to electrons of

$$\eta_{photosystem} = \frac{0.6\text{eV}}{1.83\text{eV}} = 0.33$$

This efficiency applies to each PSI and PSII—since the electrochemical potential difference required at standard state to form H_2 and O_2 from H_2O is 1.23 V. Hence, the energetic inefficiency in the photosynthetic reaction centers requires that a second photon be converted into another 0.7-0.8 eV charge-separated electron-hole pair, and the total available electrochemical potential, when the two photosynthetic processes are connected in series in the membrane, is 1.4-1.5 V, sufficient to thermodynamically drive the metabolic processes and additionally to support a pH gradient across the membrane.

The energy loss in the photosynthetic reaction centers is relatively large compared to (for example) the loss in an inorganic semiconductor such as Si or GaAs across the space-charge region of a p-n junction. Here an energy loss of only about 0.3 V is necessary to achieve similar charge separation lifetimes at near-unity quantum yields. Hence, in principle, it should be possible to design another type of photosynthetic reaction center which loses less energy in the charge-separation process. For example, a photovoltaic cell with a band gap of 1.8 eV could produce an open-circuit voltage of 1.4 V under

1 Sun illumination and run at maximum power at 1.2 V. Such a system would not require a series connection to a second PV/band gap system, and therefore if wired up to the same enzyme catalysts that are now used in the biological system, increase the efficiency by a factor of two. The energy conversion efficiency of such a PV system with a 1.8eV band gap could be about 27%.

On the other hand, it must be recognized that redesigning the photosystems to meet these requirements is not very realistic given current scientific knowledge; for all practical purposes the energy conversion efficiency of the photosystems should be regarded as fixed at the present time. It is worth keeping in mind however that the energetic efficiency of these proteins not as good as those found in modern solar photovoltaic technology.

6.3.2 Light harvesting

Thus far we have outlined two constraints associated with the basic conversion of light to electrons. The first is that the solar spectrum itself, coupled with the basic requirement that photons of wavelength 680nm and 700nm are required to drive PSI and PSII, leads to a 37% efficiency. Secondly, the conversion efficiency of the photons to electrons within each photosystem leads to an additional efficiency reduction by a factor of about 33%. One other factor associated with capturing light that we have not mentioned is the absorption efficiency – it turns out that about 95% of the light is absorbed and only 5% percent reflected. As described above, although one could 'in principle' imagine doing better in the photosystem conversion efficiency, these numbers are for all practical purposes essentially fixed—leading to an upper bound on the overall efficiency of $33 \times 37 \times 95 = 11\%$.

Biology however provides yet further constraints on the light conversion efficiency. One of the most discussed is the experimental fact that if one measures the rate of photosynthesis as a function of solar flux, the rate saturates at a critical light intensity. Figure 9 shows such data for both sun and shade plants: the sun plants achieve a severalfold increase in the maximum rate of photosynthesis. Above a critical light intensity (≈ 0.2 solar flux for sun plants and ≈ 0.1 solar flux for shade plants) the photosynthetic rate does not increase with light intensity. Such behavior is ubiquitous in photosynthetic organisms, from plants to bacteria to algae. The molecular

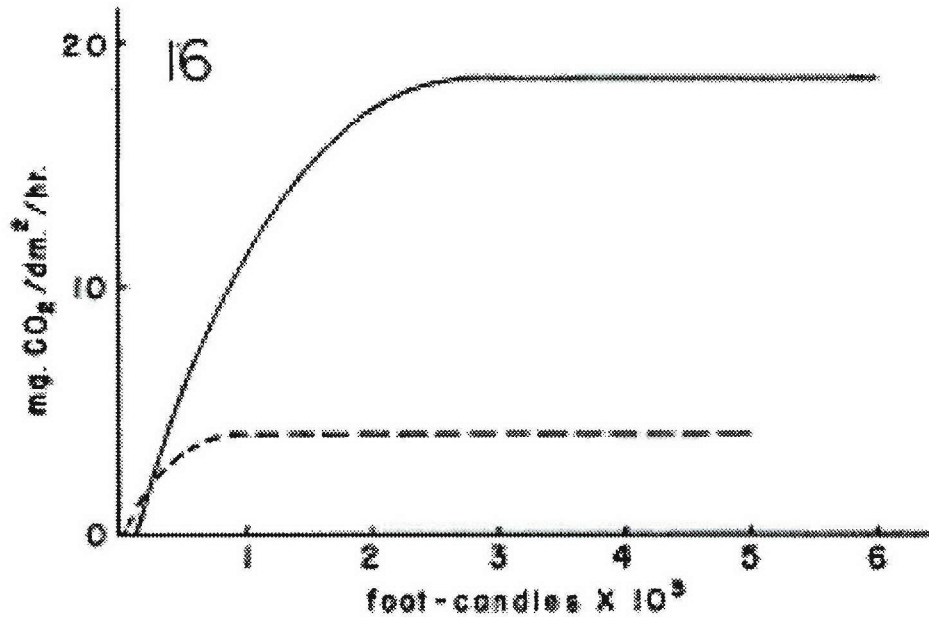


Figure 9: Comparison of photosynthetic rate as a function of incident light intensity for sun and shade plants. From [5].

reason for this behavior is that light for each photosystem is captured by a system of antenna chlorophylls. For each photosystem there is some number N of antenna chlorophylls. Now, as we have described above, the rate at which photosynthesis operates is set by the diffusion time of the plastocyanins

from PSII to PSI, which takes of order $T = 10^{-3}\text{sec}$. Any light that is fed from the antennas to the reaction center faster than this timescale will be wasted because the reaction center is busy processing the last electron. If $F = 10^{17}\text{photons/cm}^2$ is the incident solar flux, and $\sigma \approx 10^{-15}\text{cm}^2$ is the cross section of each solar flux, then the number of antenna chlorophylls N^* needed to keep up with photosynthesis is

$$F\sigma N^* = \frac{1}{T}. \quad (6-8)$$

or

$$N^* = \frac{1}{TF\sigma} \approx 10. \quad (6-9)$$

Thus under this solar flux, about a fraction of about N^*/N of the absorbed light is wasted and transferred to heat.

In practice, the number of antenna's per reaction center is of order 100–200, so that the conversion efficiency from the antennas is about ten percent. A more careful estimate can be obtained by averaging the photosynthetic yield over a solar day; if we assume that the photosynthetic rate is given by

$$P = k \frac{BF}{B + F}, \quad (6-10)$$

where F is the local intensity of sunlight and B is the saturation flux. The solar flux $F = A \sin(\pi t/T_{\text{day}})$ changes from $0 \rightarrow A \rightarrow 0$ during the course of a day. We can thus ask how the daily average rate of photosynthesis depends on the ratio of the saturation flux to the maximum solar flux, $\mu = B/A$. Figure 10 shows the result of this calculation: For $\mu = 10$, the efficiency is ≈ 0.13 .

Hence combining the light harvesting efficiency by that imposed by physical constraints (the solar spectrum) with the factors that control the operation of SI and PSII, we have an efficiency of $13\% \times 11\% \sim 1.4\%$.

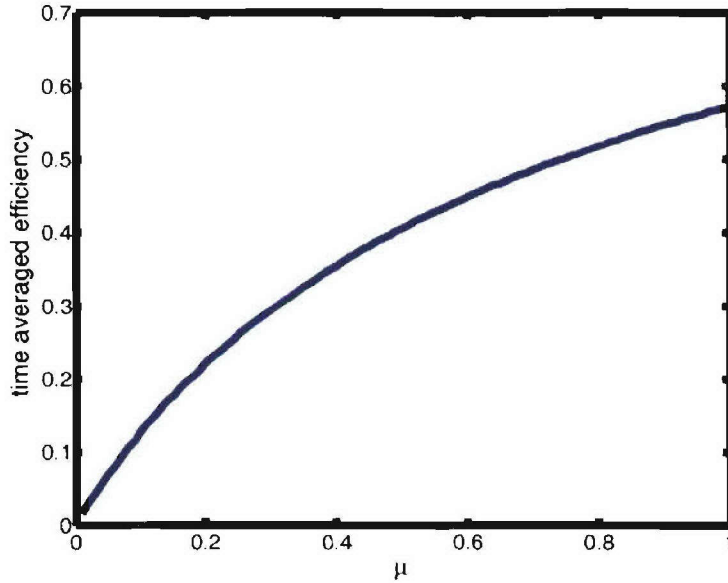


Figure 10: Photosynthetic efficiency averaged over one day, as a function of $\mu = B/A$ the ratio of the saturation flux to the maximum solar flux.

There are essentially three choices for improving the light harvesting efficiency:

1. The first option is to somehow decrease the total number of antenna chlorophylls per reaction center, in order to make the saturation point of the light curve closer to the solar intensity. This idea has been explored through the breeding of shade and sun plants (see Figure 9). Recent studies by Melis [23] in algae have received much attention in changing the light curve for photosynthetic algae *Chlamydomonas*, see Figure 11. Melis[23] performed random insertional mutagenesis and chose a mutant whose color was much less green than the wild type. The mutant indeed had a smaller antenna than the wild type, with the wild type having 240 Chl/PSI and 220 Chl/PSII, whereas the mutant had about half this number: 160 chl/PSI and 110Chl/PSII.

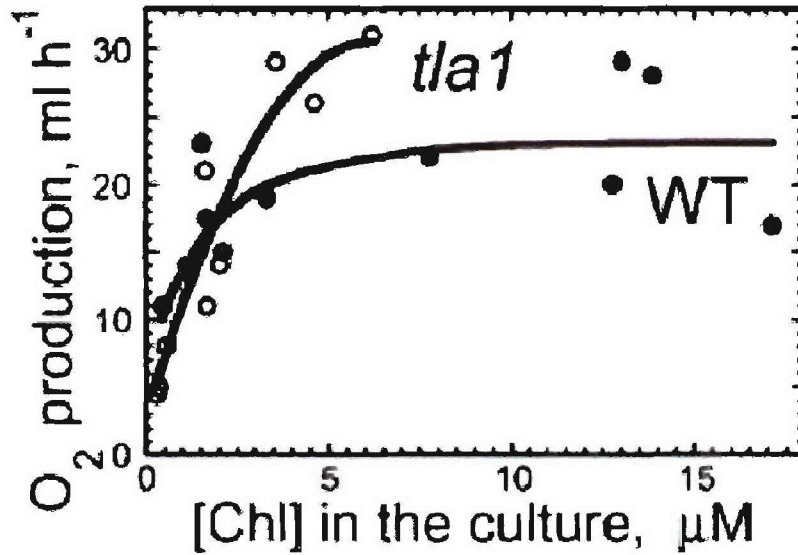


Figure 11: Photosynthetic rate for wild type (WT) versus mutant (*tla1*) (Melis et al).

On the other hand, the mutant had also about a factor of two fewer photosynthetic reaction centers per cell. The data appear to show that the photosynthetic rate indeed saturates about fifty percent higher than the wild type; presumably the photosynthetic rate did not rise by a factor of two because of the decrease in the number of reaction centers.

2. A second option would be to decrease the number N^* of antennas per reaction center when saturation occurs. The discussion above indicates that N^* depends on both T the time for the photosynthetic reaction, and σ , the absorption cross section of the antenna. One might imagine that there is some leeway to manipulate T : on the other hand, recall the discussion that the rate limiting step underlying the reaction time is the time for a plastocyanin to diffuse from PSII to PSI. This time

depends on the spatial distribution of the photosystems. We do not understand nearly enough at the present time to understand how to program the spatial distribution of the photosystems into a cell, nor do we know whether modifying the spatial distribution will cause other, perhaps deleterious, changes to photosynthesis.

3. A final option was brought forth during the JASON study, and is an entirely physical mechanism for fixing the light saturation problem. We do not know if this is original (we suspect not) but we could not find it mentioned in current literature discussions of the problem. The idea is that for either a leaf, or for algae in a pond, cells are immersed in water. Hence light that is incident normally continues in the same direction into the material. But even at grazing incidence, the light is refracted, so that it makes an angle of at least 48 deg from grazing (42 deg from normal). Moreover, near grazing incidence, the width of the beam increases dramatically from refraction: If the incident angle is near grazing so that $i = \pi/2 - G$, where G is very small, then the width of the beam increases by $0.7/G$. Hence if $G = 0.01$ radians (say), then we can decrease the solar intensity down from its value normal to the leaf to a value which is of the order of a few percent. This mechanism presumably operates in a dense patch of grass, where the leaves do not attempt to lie normal to the sun's rays, but allow the sun to be incident upon them at near-grazing angle.

It would be of interest to set up some experiments with algae beds to test this idea.

6.4 Systems Level Constraints

Thus far we have focused only on constraints due to the laws of physics and to the individual biological components that the photosynthetic apparatus uses. There are also additional constraints that come from the organization of the system itself. In the context of the computer analogy discussed above, computing power depends not just on clock speed but on how the various parts of the computer interact. These interactions often introduce constraints that would not be apparent in only examining the individual components.

During the course of our study we attempted to quantify and understand a variety of systems level constraints. Unfortunately, in essentially every case, current knowledge does not seem sufficient to nail down either what these constraints are, why they occur, or how much they affect photosynthetic yield. Here we outline a number of constraints that we have considered—there well may be more that we have not thought about.

1. **Maintenance Costs.** Part of the energy produced by photosynthesis must be used to maintain the organism and the photosynthetic apparatus. For example, the photosynthetic reaction centers must be frequently repaired from photodamage, and this costs energy. The metabolism of the organism itself requires energy. The question then is what fraction of the energy budget is used for maintenance. There is a real difficulty in obtaining measurements that address this point. Although there is a substantial literature on the energy usage in growing cells, there are essentially no measurements for the energy requirements of cells in stationary cultures, as would be used for photosynthetic microorganisms. Cell metabolism changes between these two states, with

algal species	biomass yield (low light)	maintenance cost (W/kg d. wt.) (pW/cell)		size (μm^3)
Chlorella MA003	15%	7.6	0.076*	~ 50
Chlorella 211/8k	11%	6.0	0.06*	~ 50
Chlorella 211/8k	13%	0.5	0.005*	~ 50
M. subterraneus	5%	< 7	< 0.1*	~ 50
S. protuberans	15%	130	13**	~ 500
S. protuberans	5.6%	180	18*	~ 500
Chlorogonium sp.	18%	640	64**	500 - 1000

Figure 12: Maintenance costs of various photosynthetic algae.

stationary culture presumably requiring less energy. The accompanying Figure 12 shows data that we have collected for energy requirements for different algal species (in growing culture). The species that has the closest cell size to *Chlamydomonas* is *Chlorogonium*. These algae consume about 64 pW/cell for metabolism and maintenance, but produce about 200pW/cell in photosynthetic output. Thus the maintenance cost is about 30%. Over all species we see that maintenance costs are between 5 – 30% of the maximum energy uptake rate.

2. **Light Capture Kinetics.** Many aspects of the light reactions of photosynthesis are highly regulated and co-regulated and interdependent. An example mentioned above is the dependence of N^* , the maximum number of antennas before the light harvesting apparatus saturates on T , the time of the photosynthetic reaction. The latter is set by the diffusion of the plastocyanin from PSII to PSI, a system level property. Other features are also strongly interconnected. The relative stoichiometry of PSI and PSII is regulated in two ways. First the relative sizes of the light harvesting apparatus on PSI and PSII can be dynamically ad-

justed depending on light intensity, ATP and CO₂. Two different states of the antennas have been identified—in the first most of the antenna is with PSII, and in the second most of it is with PSI. The second state is favored under conditions of low light, low ATP and low CO₂, in algae. State transitions can occur over a relatively short time. It is currently believed that state I is associated with linear electron flow, providing NADPH and ATP for CO₂ fixation, whereas state II is associated with cyclic electron flow. The transition between these two states is sensed, e.g., by the accumulation of electrons between PSI and PSII. A major point here is that given that the system is apparently set up so that there are two steady states with different antenna size requirements, it might be naive to imagine manipulating the antenna size without reengineering the entire system.

Another way that the relative stoichiometry of PSI and PSII can be adjusted is by changing the number of the reaction centers themselves. Figure 13 shows an example from Melis, comparing oxygen evolution rates in *Chlamydomonas* under low light and high light conditions. At low light intensities the saturation point is lower (due to a larger antenna), more of the antenna chlorophylls are associated with PSII, and the number of reaction centers is higher.

It should be remarked that in this example, the transition from low light conditions to high light conditions involves a simultaneous reduction in the number of reaction centers. This is similar to that observed in the *tla1* mutant. On the other hand Figure 9 shows that in sun plants both the saturation threshold and the maximum photosynthetic rate is higher. Whether this is due to improvements in architecture of the plant as a whole, or improvements in the regulatory circuits inside individual cells is an interesting question: Is it possible to si-

	chl/PSI*	chl/PSII*	PSI*/cell	PSII*/cell
Low light	250	500	2×10^6	2×10^6
High light	100	60	0.2×10^6	0.6×10^6

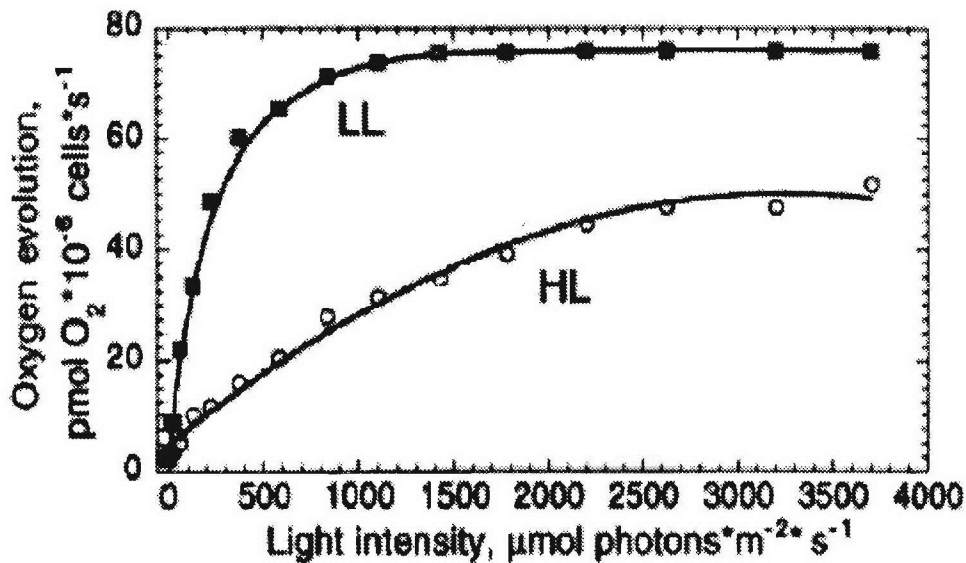


Figure 13: Transition in photosynthetic rates between low light levels and high light levels. Note that at low light the antenna is larger, more of it is associated with PSII and there are more reaction centers. From Melis 1998

multaneously increase the light saturation threshold and the number of photosynthetic reaction centers within a single cell?

Finally, it is worth remarking again at this point the fact that the timescale for the photosynthetic reaction is set by the time it takes for the plastocyanin to diffuse from PSII to PSI. The architecture of these components is a systems level property and changing this time scale (speeding it up?) is a systems level constraint. Of course, it also must be stated that whether it is useful to speed up the light harvesting part of photosynthesis depends critically on whether it is possible to speed

up the enzymatics of the dark reactions. Typical enzyme turnover times in biology are not faster than 10^{-3} sec, so that it seems possible that the rate of the light reactions is tuned to the turnover rate of the dark reactions. If this were the case, no significant improvement could be made unless the light reactions and dark reactions were sped up in parallel.

3. Coupling of Product Stream to Photosynthetic components.

Another significant systems constraint involves the coupling of the light reactions to the dark reactions, and vice versa. The dark reactions require protons and electrons, in the form of NADPH and ATP; but if these species and any excess electronics as free radicals damage the cell if they are not used. There is thus exquisite regulation controlling the light reactions owing to the needs of the dark reactions.

In all of these cases, the main question is how to quantify the constraints that the regulatory machinery place on the photosynthetic apparatus. Or, said differently, which part of the system limits the photosynthetic rate? Returning to our example of the speed of a computer, one could imagine that the actual speed of computation could be determined by any number of individual components, or by limitations set by the interactions between several of the components. For the case of photosynthesis, we do not know precisely what is providing the limit—other than the obvious constraint that the light and dark reactions are strongly coupled. In principle, the limit could come from individual components (e.g., the ideas about decreasing the size of the light harvesting apparatus) or it could come from systems level constraints. Discovering the precise bottleneck is likely to be critical for progress.

7 THE SPECIFIC CASE OF HYDROGEN PRODUCTION

Having now given a detailed summary of photosynthesis and photosynthetic constraints, we turn to a discussion of the specific case of hydrogen production by microorganisms. What is particularly interesting about this example is that bacteria and algae certainly did not evolve to produce hydrogen. Indeed, the physiological function of a hydrogen metabolism is still unknown and debated. The most common idea [1, 4] is that the hydrogen metabolism assists survival under extreme conditions. In any case, it seems clear that in present day organisms with a hydrogen metabolism, the most prominent of which are the cyanobacteria and the green algae *Chlamydomonas*, hydrogen production is not a primary determinant of the organisms's fitness. Hence, trying to engineer these organisms to scale up hydrogen production provides an excellent specific case to discuss the challenges and bottlenecks that could come up when engineering a microorganism for fuel production.

7.1 The Technical Hurdles

The technical hurdles and the state of the art for biohydrogen production have been well summarized in a number of recent reviews[4, 23, 30, 3, 20], as well as in an excellent older review[21]. Here we outline the main points and explain how they fit into our overall argument.

7.1.1 Oxygen sensitivity of hydrogenase

Hydrogen production in both cyanobacteria and algae relies on specialized enzymes to catalyze the reactions. As mentioned above, there are two possibilities. Cyanobacteria have nitrogenases, which catalyze reactions producing both NH_3 and H_2 . These reactions require an energy input of at least 2 ATP per electron. Additionally many cyanobacteria have so-called uptake hydrogenases that reconvert the electrons in H_2 , degrading the efficiency further. Even if the uptake hydrogenases could be eliminated, the energy overhead of the nitrogenase reactions makes them an unlikely competitor for producing hydrogen.

Another option for catalyzing the reaction are hydrogenases. There exist hydrogenase enzymes which catalyze the reaction without requiring any ATP. These enzymes have high specific activities, but their main difficulty is that they are extremely sensitive to oxygen—small amounts of oxygen cause them to irreversibly shut off. (Indeed, nitrogenases suffer from this same difficulty.)

Figure 14 shows the specific activity of hydrogenase enzymes from several photosynthetic algae. The laboratory work-horse *Chlamydomonas reinhardtii* is poisoned upon exposure to air in less than a second. In contrast, recent work[29, 26], has shown that the *Chlostridium* has a substantially larger half life, though still only several minutes.

The question is whether a hydrogenase can be either discovered or engineered with highly reduced oxygen sensitivity. Otherwise, the hydrogen production reaction can only take place in the absence of oxygen. Indeed, the early demonstration by Greenbaum of the hydrogenase reaction in *Chlamydomonas* was done by continuously purging the oxygen out of the sample cell.

Organism	Protein Name	Subunit Composition	Activity (nMol H ₂ / ml/min)	Specific Activity (nMol H ₂ / mg/min)	Half-life after exposure to air
<i>Chlamydomonas reinhardtii</i>	HydA1	Monomeric	42	212	< 1 sec
<i>Chlamydomonas reinhardtii</i>	HydA2	Monomeric	12	708	< 1 sec
<i>Clostridium acetobutylicum</i>	Cal	Monomeric	28	2894	415±115
	CalI	Monomeric	3	682	
<i>Clostridium pasteurianum</i>	Cpl	Monomeric	20	ND	120-300
<i>Shewanella oneidensis</i>	HydAB	Dimeric	14	850	

Figure 14: Table summarizing the hydrogenase enzymes of various photosynthetic algae, the activity of the enzyme and its half life upon exposure to air (From M. Ghiardi briefing.)

The pumping costs associated with this would severely reduce the efficiency of the process.

There are reasons for both optimism and pessimism in the quest for an oxygen insensitive hydrogenase. On the optimistic side, substantial progress has been made recently in understanding the structure and hence the mechanism of operation of the hydrogenases, and this has led to both careful thinking and modelling (e.g., we were briefed in this regard by M. Ghiradi) for why the enzymes are oxygen sensitive. Such understanding will lead to a natural opportunity for the possible rational redesign of the enzymes.

Another reason for optimism is that the number of photosynthetic algae that have been carefully studied is relatively small; recent efforts of Venter and collaborators could lead to the discovery of new algal species and associated hydrogenases that are markedly less sensitive to oxygen. The fact that in Figure 14 *Clostridium* has a 400-fold higher half life than *Chlamydomonas*

well illustrates the opportunity. Are there organisms with even higher half lives?

The main reason for pessimism is that there is presently no known chemical system, living or nonliving, that can evolve hydrogen without reacting with oxygen. Although we know of no fundamental principle that says such a system cannot exist, we also do not know of any principle that says such a system can exist. It is perhaps worth recounting here the experience of evolution with rubisco, the central enzyme in the dark reactions of photosynthesis. Rubisco also reacts with oxygen, leading to photorespiration. C4 plants managed to limit photorespiration by evolving a compartment where the oxygen concentrations are much lower than that of C3 plants; it did *not*, however find a way to re-engineer the enzyme.

7.1.2 Competition with the Calvin cycle

A second major difficulty with the efficiency of hydrogen production is that the hydrogenase reaction competes with the Calvin cycle. If CO₂ is present, electrons (NADPH) will be used to facilitate the production of carbohydrate and thus decrease the yield of H₂. In order for efficient hydrogen production to proceed, the Calvin cycle must be inhibited: either by depriving the system of CO₂ or by disabling it in another way. On the other hand, it would presumably be detrimental to the system to completely shut off the Calvin cycle, as this would prohibit any growth or regeneration of cells.

7.1.3 Proton transport

Like the Calvin cycle, the hydrogenase reaction takes place outside of the thylakoid membrane. The proton concentration outside the thylakoid mem-

brane must be large enough to react with all (most) of the electrons produced by photosynthesis. In the absence of other sources of protons, this would require that *all* of the protons that are produced by the water splitting (inside the thylakoid membrane) move to the outside of the thylakoid membrane to react with the photosynthetically produced electrons.

On the other hand, the proton gradient across the thylakoid membrane is the primary driver of the ATP synthase, which drives the production of ATP. Hence any effort to move the protons outside the thylakoid membrane will affect the ATP/NADPH balance of the cell.

This tension between ATP production on one hand, and the need for the protons to mainly live outside the thylakoid membrane for efficient production of H_2 exposes one of the primary tensions of biohydrogen production by microorganisms. If all of the protons are moved to the outside of the thylakoid membrane, no ATP will be produced. But presumably, the organisms need ATP to live, and to maintain and repair the photosynthetic machinery. How much energy this requires, in practice or in principle, is an interesting question whose answer we could not find in the literature (See the discussion above on maintenance costs).

7.1.4 Producing hydrogenase

The hydrogenase enzyme is not produced under normal situations. Classically, the enzyme is produced in the dark, when the culture is deprived of oxygen. However the genetic circuits underlying this switch are not known. If for example the promotor for the hydrogenase enzyme were discovered, it could be possible to turn on production of the enzyme under normal conditions. In partial support of this, recent work by Ghirardi, Melis and collabo-

rators discovered a mechanism for expressing the hydrogenase enzyme in the light.

7.2 State of the art Experiments

The JASON's were briefed on several experiments demonstrating the production of hydrogen from algal cultures. The experiments used a range of strategies to get around the aforementioned challenges, ranging from

1. Anerobiosis, by pumping the O_2 out of the system. This solved the problem of the oxygen-sensitive hydrogenase (by getting rid of the oxygen.)
2. Limit the CO_2 supply.
3. Limit protein synthesis, by for example sulfur deprivation.
4. Increase the proton conductance of the thylakoid membrane to increase the proton concentration outside the membrane.

7.2.1 Sulfur deprivation

The first experiment we discuss is that by Melis *et al.*, [25, 13, 24]. The authors discovered that by depriving the growth medium of sulfur they could induce the production of hydrogenase enzyme and begin production of hydrogen. The experiment works because sulfur deprivation impedes protein biosynthesis, which thereby blocks the regeneration of photosystem II. This causes the rate of photosynthetic oxygen production to drop below the rate of oxygen consumption by respiration. Hence, the culture becomes anaerobic in the light. The organisms then turn on the hydrogenase enzyme, and begin to

produce hydrogen. Figure 15 demonstrates this process. Figure 15A shows the rate of photosynthetic production of oxygen, and the rate of consumption of oxygen by respiration, after sulfur deprivation. Twenty five hours after deprivation, the respiration rate is larger than the production rate, and anaerobiosis begins. Shortly thereafter (Figure 15B) hydrogen production starts. After about 100 hours, the rate of production of hydrogen levels off, presumably because of photodamage to the photosynthetic machinery. At this point it is necessary to reintroduce sulfur into the growth medium and let the culture regrow and regenerate its photosynthetic machinery. Melis *et al.* demonstrated that they could cycle the system from a sulfur rich state to a sulfur deprivation state. There is some current controversy about whether this experiment really represents indirect photobiolysis, or whether consumption of the growth medium (specifically acetate) is important for the process [30].

What is the efficiency of energy production in this experiment? If we examine only the phase where hydrogen gas is collected, the experiment produced $\approx 120\text{ml}$ of H_2 gas in about 90 hours. The collection was done in a one liter flask, so we might approximate the illumination area as 100cm^2 . The incoming light intensity was about 200μ moles photons/ m^2/sec . At atmospheric pressure, hydrogen gas has an energy density of $10.7\text{MJ}/\text{m}^3$. Hence, the gas has an energy of $120 \times 10^{-6}\text{m}^3 \times 10.7\text{MJ}/\text{m}^3 = 1284\text{J}$. The energy output is therefore $1284\text{J}/(90 \times 3600 \text{ sec})/(0.1 \text{ m})^2 = 0.39\text{W}/\text{m}^2$.

If we assume the input photons have wavelength 600 nm, the input energy is $\approx 40\text{W}/\text{m}^2$. Putting this together, the overall efficiency is about 1%. Of course we also must discount this by the energy input during the time that it takes to repair the photosynthetic apparatus, which leaves us with an efficiency of $\approx 0.5\%$.

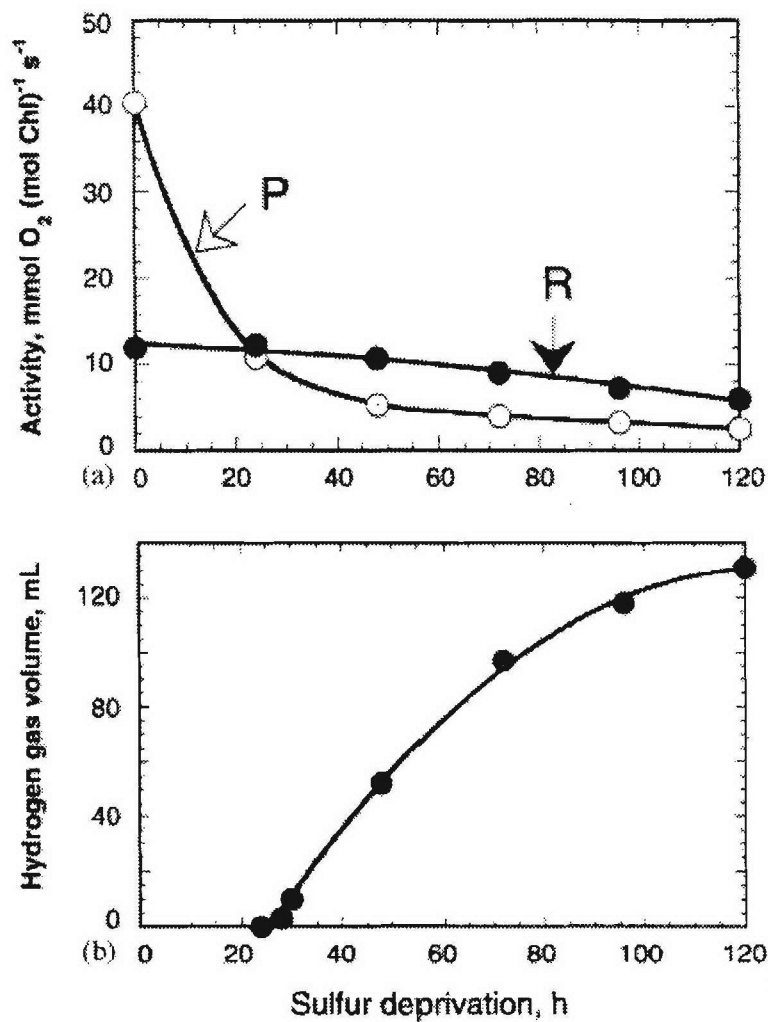


Figure 15: (A) rate of photosynthetic production of oxygen, and rate of consumption of oxygen by respiration, after culture is deprived of sulfur. At around 25 hours after deprivation the respiration rate is larger than the production rate which leads to anaerobiosis. (B) Shortly thereafter, hydrogen production commences. From [25].

This is a surprisingly high efficiency, especially given the evidence in Figure 15 that sulfur deprivation cuts the normal photosynthesis rate by a factor of twenty. From our discussion above, one would conclude that the maximum efficiency of photosynthesis is at best $\sim 1\%$ (though in practice it is much lower, see discussion above); a twenty fold decrease in the efficiency represented by a decrease in the rate of photosynthesis would therefore lead to the efficiency of about $1\%/20 = 0.05\%$. This estimate assumes that all of the absorbed photons are used for hydrogen production, with no additional losses. The source of this discrepancy between the calculation in the previous paragraph and that given here is unclear.

7.2.2 Simultaneous production of H_2 and O_2 in a confined reactor

Another recent experiment [14] demonstrating hydrogen production was done by Greenbaum and collaborators; the hydrogenase enzyme was first induced in a dark, anaerobic environment. During the light reaction the system was continuously purged of CO_2 in order to inhibit the Calvin cycle. Figure 16 shows the evolution of both hydrogen and oxygen in this experiment. Each data point corresponds to one hour of illumination, but between every two data points there is a 2 hour purge in the darkness to completely remove hydrogen and oxygen from the system.

What is the efficiency of this hydrogen production? Let us neglect the energy used to pump the oxygen and hydrogen out of the system. The experiment produces about $40\mu\text{moles } H_2/\text{hr}/(\text{mgchl})$. The container contains about $200\mu\text{g}/\text{chl}$, so the production rate of hydrogen is $8\mu\text{ moles/hr}$. If we again take the illumination area as 100cm^2 , this corresponds to an energy of $0.03\text{W}/\text{m}^2$. Now the input illumination is $150\mu\text{moles}/\text{m}^2/\text{sec}$ of photons in the wavelength range $400 - 700\text{nm}$. This corresponds to an input energy of

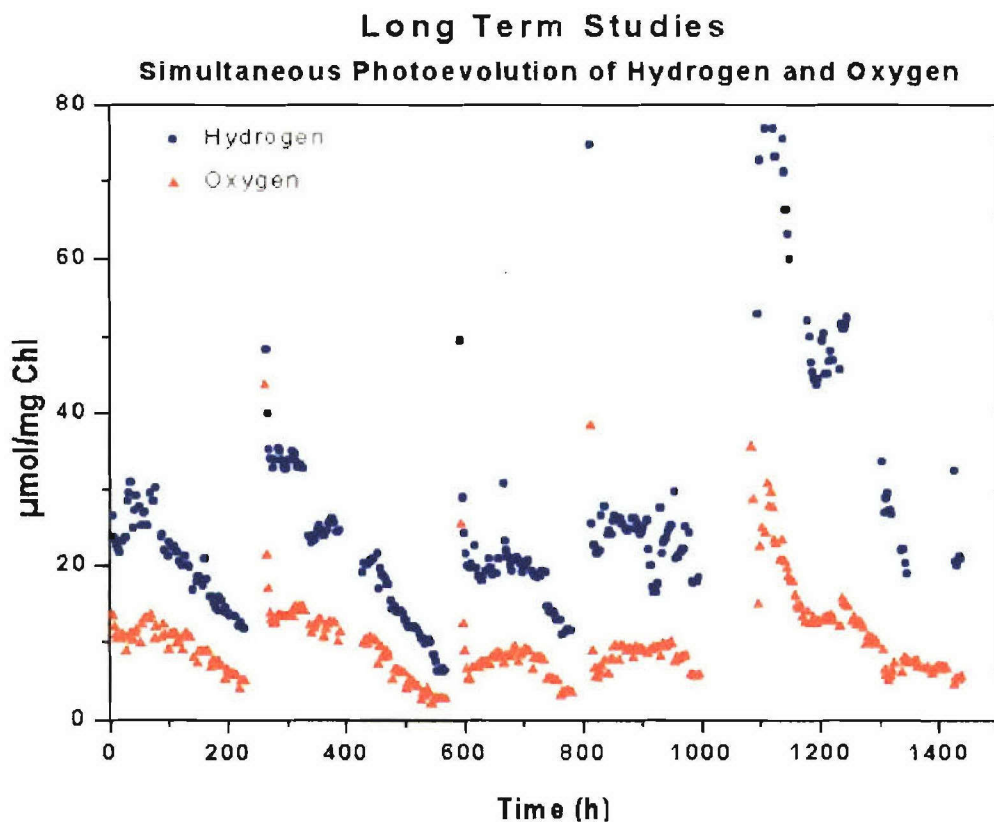


Figure 16: Production of both hydrogen and oxygen. (E. Greenbaum Briefing.)

$\sim 60\text{W/m}^2$, giving an efficiency of 0.05%, where again we have neglected the role of pumping costs.

7.3 Engineering Issues

It is worth remarking briefly on some of the engineering issues and challenges that would come up were hydrogen production by microorganisms ready for large scale production.

1. The hydrogen gas would be collected as a mixture together with oxy-

gen. as both bubble out of the solution (See Figure 15). Hydrogen must be separated from oxygen at the source because their mixture is explosive and hazardous, and cannot be safely transported or stored. The separation of hydrogen from oxygen bears some resemblance to that of nitrogen from oxygen, which is done on a large industrial scale in the production of oxygen or of liquid nitrogen from air, in that both components are comparatively unreactive and gaseous homonuclear diatomic molecules. However, there is little experience with the separation of hydrogen from oxygen because extant (non-biosynthetic) production of hydrogen is by electrolysis, in which hydrogen and oxygen are produced at opposite electrodes, and are not mixed, or by chemical reactions (such as the reaction of acids with metals) in which hydrogen is the only gaseous product.

How much energy will it cost to separate the hydrogen and oxygen? Present industrial separation of nitrogen from oxygen is based either on differential adsorption (pressure swing adsorption or vacuum swing adsorption), differential membrane permeability, or differential vapor pressure (distillation). The entropy per molecule required to separate a mixture of ideal gases consisting of a fraction α of one component and a fraction $1 - \alpha$ of a second component is

$$ds = -k_B(\alpha \ln \alpha + (1 - \alpha) \ln 1 - \alpha).$$

For a stoichiometric mixture of H_2 and O_2 this is $0.637k_B$ per molecule of the mixture. The ideal free energy required to accomplish this separation at 30°C is

$$dF = TdS = TN_A ds = 2.40 \times 10^3 \text{ J/mole}.$$

This should be compared to the enthalpy of combustion of H_2 , which is $2.86 \times 10^5 \text{ J/mole}$ (to the liquid state), about 120 times greater.

Of course, separation cannot be performed at its ideal thermodynamic efficiency. Unfortunately, realistic estimates of attainable efficiency are not easily obtained, even for the industrial nitrogen-oxygen separation, for which they are considered proprietary. Plausible guesses are that the efficiency is 0.2–0.5 times its thermodynamic limit. In addition, the enthalpy of combustion cannot generally be converted to a useful form with unit efficiency; typical efficiencies of making electricity from combustion energy are in the range 40–50%, although fuel cells should do better. Combining these factors implies that hydrogen separation exacts, at most, as 3–10% reduction in the energy productivity of biosynthetic hydrogen production.

As a pessimistic estimate, suppose that hydrogen-oxygen separation can only be done by cryogenic condensation of the oxygen, and moreover the process is maximally irreversible (no heat exchangers between incoming and outgoing streams). The latent heat of boiling of O_2 is 6.82×10^3 J/mole, or 3.41×10^3 J/mole of H_2 , requiring the removal of an entropy per molecule of H_2 of $ds = 4.57k_B$ at $90^\circ K$, the normal boiling point of O_2 . Cooling the gas mixture from $T_h = 30^\circ C = 303^\circ K$ to $T_c = 90^\circ K$ requires the removal of an entropy per molecule of H_2 of $ds = \frac{3}{2} \ln T_h/T_c k_B = 4.55k_B$ (the first factor allows for the fact that only 2/3 of the molecules are H_2 , and we have assumed a classical model for the rotational specific heats while neglecting the vibrational specific heats; the classical model of rotational specific heats permits us to neglect ortho-para effects). Combining these two terms gives us $ds = 9.12k_B$, corresponding to

$$dF = TdS = TN_A ds = 2.29 \times 10^4 \text{ J/mole}$$

at $30^\circ C$. This is about 8% of the enthalpy of combustion, or 16–20% of

the free energy obtainable from combustion in a thermal power plant. We have unrealistically assumed ideal efficiency for the cryogenic refrigerator, but this is offset by our assumption of maximal irreversibility (maximally bad design) of the cooling and liquefaction processes. Hence it is fair to conclude that even if separation must be by liquefaction, it will not impose a prohibitive tax on the energy content of the produced hydrogen.

2. A critical engineering issue is to understand the "systems cost" of energy production by microorganisms: How much would it cost (per unit area) to run and maintain an algal farm designed to produce energy? Such an analysis should include not only the hydrogen/oxygen separation costs described above, but also how the gas would be transported to (presumably) a central facility where liquid fuels could be created. A careful systems level analysis is critical for predicting the ultimate success of this technology. A preliminary study including some of the important factors was carried out in [2], inspired by the sulfur deprivation experiments of Melis *et. al.* described above.

8 STRATEGIES FOR IMPROVING AND PRODUCING NEW FUELS

As discussed above, there is a substantial gap between the currently attainable bioenergy yield and the estimated upper bound of $\sim 10\%$ based on the known properties of the components of the photosynthesis machinery. In this section, we examine various aspects of the known photosynthesis pathway in order to identify bottlenecks and explore strategies to improve the energy yield. We will illustrate the issues in the context of biohydrogen evolution, which is a simpler conceptual problem and is also somewhat more distinct (compared e.g., to bioethanol production) from issues addressed in the traditional metabolic engineering context.

8.1 Hydrogen Evolution as an Application of Metabolic Engineering

It is desirable for hydrogen evolution to occur in aerobic conditions in order to minimize the investment of extra energy needed to stringently maintain anaerobic conditions. This task is akin to those routinely encountered in metabolic engineering, where a specific chemical compound, e.g., amino acids or carotenoids, is to be produced in large quantities. Such metabolic engineering tasks typically consist of several key challenges:

1. to increase the input flux (in this case, photosynthetic electrons)
2. to increase the efficiency of product synthesis (in this case, hydrogenase activity),
3. to re-route the input flux towards product synthesis.

Metabolic engineers use a variety of methods to meet these challenges, including directed evolution of key molecular components, combinatorial, and rational design of alternative metabolic pathways. Below, we will describe these methods in the context of the hydrogen evolution problem. Conceptually, hydrogen evolution is simpler than canonical metabolic engineering applications in that the terminal pathway is of only one step, which branches immediately from the main input pathway. On the other hand, the photosynthesis pathway is highly regulated, as organisms generally transduce just enough energy to satisfy their metabolic needs. Moreover, compared to most biosynthetic pathways, regulation of the photosynthetic pathways is not understood as well and involves molecular components (e.g., membrane proteins and co-factors) that are not as easy to manipulate by genetic means. The discussion below serves both to illustrate applications of possible metabolic engineering strategies and to expose problems specific to photosynthesis and hydrogen evolution.

8.1.1 Increasing the yield of photosynthetic electrons

As discussed above, a major obstacle preventing the more efficient capture of photoenergy is the saturation of the photosynthesis rate for incident irradiation beyond 0.1–0.2 solar flux. This effect is attributed to the slow electron current out of PSII, which limits the ability of the reaction center to convert the photoenergy captured by the chlorophylls into additional electron current. As reengineering the Photosystems themselves is beyond the reach of current knowledge and abilities, strategies to overcome this obstacle have centered around ways to avoid light saturation. These include the physical method to dilute the incident irradiation, and the genetic approach to reduce the antenna size (the number of chlorophyll molecule per reaction

center) as explored by Melis et al [28] and discussed above. By distributing the incident irradiation to a larger number of cells, these methods aim to improve the overall yield of photosynthesis by reducing the photoenergy waste (the photoenergy that chlorophyll absorbs but the reaction center cannot process is dissipated into heat). However, they do not address the specific yield (rate of photosynthetic electron production per cell), which determines the size of the culture that needs to be actively maintained. This may be the appropriate strategy for the time being, as the current bottleneck for hydrogen evolution is the conversion of photosynthetic electron current to hydrogen, rather than the electron current itself (see below). However, as hydrogen evolution becomes more efficient, it will eventually be useful to find ways to improve the specific yield of photosynthetic electrons. This may be accomplished in principle by increasing the number of reaction centers which are fitted with smaller antenna sizes. In practice, this will not be an easy task as little is known about genes and regulatory mechanisms that control the number of reaction centers and the size of the antennas. Basic molecular biology research is needed here before engineering efforts can be attempted. Needed here are strong selectable markers that can be used to identify the number of reaction centers and the antenna sizes. For example, the Melis lab recently identified the *tlal* mutant of *Chlamydomonas* based simply on differences in shades of greenness shown by the different mutants. By adopting more quantitative optical characterization in a high throughput capacity, it should be possible to screen a much larger number of mutants with desired reaction centers and antenna sizes.

8.1.2 Increasing the efficiency of hydrogenase activity

A potential bottleneck of hydrogen evolution is the activity of the [Fe]-

hydrogenase: In principle, does the hydrogenase produce H_2 quickly enough to process photosynthetic electrons? The specific activity of the hydrogenase was measured to be $\sim 100\text{nmolH}_2/(\text{mg} - \text{min})$ (M. Ghirardi briefing³) Let us assume a cell mass of 10^{-9} g and $\sim 10^6$ reaction centers per cell: if the hydrogenase is say $x\%$ of the cell mass, this corresponds to ~ 1 H_2 molecules evolved/sec per reaction center, about $10^{-2}x$ of the rate of photosynthetic electrons generated by the Photosystem at maximum capacity. Hence, hydrogenase with this level of activity can apparently only keep up with photosynthetic electron production if of order the entire cell mass is filled with hydrogenase. Moreover, a glaring shortcoming of the hydrogenase of *Chlamydomonas* is its extreme sensitivity to oxygen, with a half-life of < 1 sec when exposed to oxygen. Interestingly, a bacterial [Fe]-hydrogenase (*Clostridium Pasteurianum*) was shown to be two orders of magnitude more stable in the presence of oxygen, although the half-life itself was still quite short (< 10 min). In vitro and in vivo coupling of the clostridial hydrogenase with the cyanobacterial photosynthetic system via cyanobacterial ferredoxin was demonstrated in the presence of light [ref: asada99]. Recently, H_2 evolution by the [NiFe]-hydrogenase of cyanobacteria has also been studied. A *Synechocystis* strain deficient in its native NADPH-dehydrogenase complex was shown to evolve a significant amount of H_2 in light [9]. Significantly, an O_2 -tolerant [NiFe]-hydrogenase from *R. gelatinosus* was recently identified [26] with a half-life of 21 hours in vivo and 6 hours in purified form in vitro. In vitro experiments demonstrated that this O_2 -tolerant [NiFe]-hydrogenase could work with ferredoxin of red algae as the electron donor, although at a diminished activity level.

Currently, there is an effort at NREL to reengineer the hydrogenase of *Chlamydomonas* to make it more O_2 -tolerant [26]. The work is computa-

³Here the activity is measured per unit purified protein.

tional, based on homology modeling of the [Fe]-hydrogenase of *Clostridium Pasteurianum* whose crystal structure is available.

Directed Evolution of Hydrogenase Given the availability of hydrogenases with improved O₂-tolerance in various organisms, we suggest that directed evolution may be an effective way to find the desired hydrogenase with both O₂-tolerance and strong coupling to the ferredoxin of *Chlamydomonas* to yield high specific activity. Directed evolution is an iterative scheme of generating genotypical variations and selecting for those with a desired phenotype. This principle has been used successfully in breeding animals and plants throughout the history of mankind. It has also been used in metabolic engineering to obtain enzymes with various desired properties [19, 10]. In laboratory-scale protein evolution, genotypical variations are typically generated by random mutagenesis and/or recombination, followed by screening or selection [34]. The applicability of the directed evolution approach is dependent largely on the existence of a powerful selection scheme or a high-throughput screening assay. It is especially useful if the selection/screening assay can identify small changes in phenotype so that the corresponding mutants may be amplified in subsequent rounds of evolution. Recently, Posewitz et al (2005) [29] showed the feasibility of high-throughput screening of H₂ production in *C. reinhardtii*, by using a library of 6000 colonies on agar plates with sensitive chemochromic H₂-sensor films. Such methods enable the application of directed evolution methodology to finding improved hydrogenases in *C. reinhardtii*.

The effectiveness of the directed evolution approach is dictated to a large extent by the size of the viable mutant pool. On the one hand, it is desired for the population to acquire as much mutation as possible, as evolution is driven by the variability of the population. On the other hand, too

large a degree of mutation tends to make most mutants not viable. This conundrum is addressed to a large degree by the method of DNA shuffling [32, 33], which randomly fragments a population of homologous DNA sequences and then reassembles them into full-length, chimeric sequences by PCR. The idea is to increase the diversity of the viable population by mixing a starting pool of proteins that have been proven to work through natural selection. Over the decade since it was introduced, DNA shuffling (combined together with random mutagenesis by error-prone PCR) has contributed to dramatic increases in the efficiency with which large phenotypic improvements are obtained. We believe the existence of a large number of hydrogenases from bacterial and alga species with varying degrees of performance (ranging from high specific activity but O_2 -sensitive to low specific activity but O_2 -tolerant) make this problem well suited for directed evolution by DNA shuffling. Two independent approaches may be adopted: One is to evolve the [Fe]-hydrogenases for improved O_2 -tolerance, the other is to evolve the O_2 -tolerant [NiFe]-hydrogenase for increased activity with the *Chlamydomonas* ferredoxin. The initial phase of either approach may proceed in vitro, which allows exploitation over a larger library ($10^3 - 10^6$ for high-throughput screening and $> 10^{12}$ for display methods [31]). Eventually, iteration of in vitro shuffling/mutagenesis and in vivo selection may be used to ascertain the effectiveness of the in vivo function while still imposing a high mutation rate. For the purpose of increasing the efficiency of hydrogenation, it may be useful to evolve both the hydrogenase and the ferredoxin. In this case, it is important to maintain the selection in vivo to ensure that the mutated ferredoxin functions properly with the rest of the electron transfer system of the photosynthetic pathway.

8.2 Re-routing the Photosynthetic Electron Current

The end product of the photosynthetic electron transfer system is the reduced ferredoxin (Fd^*). It is normally used to charge the canonical electron carrier NADPH, which together with ATP fuels the Calvin cycle. Until a time when the first two goals above can be accomplished, i.e., an increased number of reaction centers with truncated antenna sizes is installed and an oxygen-tolerant hydrogenase with high specific activity for hydrogen evolution can be expressed in large quantities, it will be necessary to limit the electron flux to the Calvin cycle in order for there to be any significant flux for hydrogen evolution. Below we will address the Calvin cycle flow first, while noting that according to the analysis in Section 6.2, CO_2 flux will be a natural limiting factor if the photosynthetic electron current can be increased substantially, through more efficient light harvesting. Afterwards, we will address the regulatory issues to insure that the reduced demand for electron flux by the Calvin cycle leads to an increased flux into hydrogen evolution rather than an overall repression of the photosynthetic electron current.

8.2.1 Limiting the electron flux to the Calvin cycle

The demand for photosynthetic electron current arises primarily from the need to reduce CO_2 in the Calvin cycle. One straightforward way to reduce this demand is to reduce the CO_2 partial pressure in the environment. As this may be energetically costly for large-scale implementations, we discuss various genetic strategies.

Reduction of CO_2 uptake. CO_2 loss via diffusion across the cell membrane is a potentially serious problem for unicellular organisms such as

algae. A strategy the algae adopt to overcome this problem is to convert the CO_2 into HCO_3^- which does not diffuse easily across the membrane, due to its charge. HCO_3^- is actively sequestered by Na^+ -dependent and other transporter systems, and is converted back to CO_2 by the enzyme carbonic anhydrase, at the site where Rubisco is packed. This ATP-consuming process effectively increases the affinity of Rubisco for CO_2 sufficiently for CO_2 fixation. Given the knowledge of this pathway, one can in principle reduce the CO_2 uptake rate by reducing the expression of key enzymes in this pathway; see below. Of course, disabling the Calvin cycle itself is another way to turn down the demand for photosynthetic electrons. This may be done by reducing the expression of genes encoding key Calvin cycle enzymes, downregulating the electron flux to NADPH. Merely reducing the Calvin cycle current may lead to an elevated level of NADPH, which may have other undesirable effects, e.g., reduction of the photosynthetic current itself, through negative feedback. Since NADPH is the designated end product of the photosynthetic electron current, downregulating its conversion from Fd^* is the most direct way of reducing the competing electron flow. This can be done by reducing the expression of FNR which catalyzes the transfer of electrons from Fd^* . A diminished NADPH level resulting from a reduction in the rate of electron transfer from Fd^* to NADPH also has the added benefit of naturally reducing the activity of some Calvin cycle enzymes that use NADPH as their allosteric activator.

8.2.2 Effect of the redox-dependent feedback

The photosynthetic system has an intricate set of feedback controls which ensures that in the situation of low light and low CO_2 , the Calvin cycle is turned off and the captured photoenergy is directed primarily into

ATP synthesis, while in the situation of high light and high CO_2 , the Calvin cycle is turned on and ATP synthesis is maintained at a level to support the demand by the Calvin cycle[8]. To understand the effect of re-routing the photosynthetic electron current from the Calvin cycle to hydrogen evolution, it is crucial to understand the feedback system that gives rise to the robust two-mode behavior.

To illustrate this effect, we describe below two possible models of this feedback control; both are based on the known facts reported in the literature, and both result in very different conclusions for understanding the consequence on the re-routing of the electron current. Without understanding enough about the regulation to understand which model is correct it is impossible to proceed with confidence.

Model 1 In this model, the two-state nature of the photosynthetic system is realized in two distinct modes of electron flow. As described above, the linear flow corresponds to the electrons generated from PSII are directed via the electron transfer system and PSI to reduce ferredoxin and ultimately NADPH. This pathway prevails in the high light situation. The cyclic flow describes the alternative situation in which few electrons are generated by PSII; instead, the energetic electron (excited by PSI) is transferred from the reduced ferredoxin (Fd^*) to plastoquinone (PQ), and then recycled through the electron transport system. In this case the energy of the electron absorbed from PSI is used to pump protons into the lumen and synthesize ATP.

For a given environment, the actual mode of electron flow is selected by the redox potential, indicated by the level of Fd^* . As will be argued below, the feedback regulation is such that the system supports either a high or low level of Fd^* . Fd^* is a powerful reducing agent; a high level of Fd^* activates regulators such as ferredoxin-thioredoxin reductase (FTR) and thioredoxin

(Td), which in turn activates both the expression and the activity of a large number of Calvin cycle enzymes. Thus, the redox level controls the carbon flux through the Calvin cycle. When the redox level is low, the Calvin cycle enzymes are not activated. This shuts off the carbon flux, and also the linear electron flow. On the other hand, at low redox level a state transition is known to occur, where up to 85% of the light harvesting complex LHCII switches association from PSII to PSI . This transition virtually shuts off PSII. The few electrons generated are fed into the enlarged pool of PSI to generate Fd^* . Since the flow to the Calvin cycle is shut off at low Fd^* , the electron current recycles from Fd^* back to PQ, thereby completing the cyclic flow. Thus the transition between linear and cyclic electron flow is driven by the state transition in Chlamydomonas [18, 12].

Crucial to this model of alternate electron flow is the coordination of the state transition and the transition in Calvin cycle activity, both controlled by the redox potential. The occurrence of a state transition requires the phosphorylation of LHCII by LHCII kinase (LK). LK is activated by a surplus of the reduced form of PQ (PQ^*) when Fd^* level is low. When the redox level is high, however, LK is inactivated by the reduction of its disulfide bond; consequently, the state transition can no longer occur even if the PQ^* level is high. The regulatory effects described above are summarized in Figure 17 (red lines). A key feature of this regulatory circuit is an effective positive feedback of the level of Fd^* on the linear electron flow, mediated through the double negative effect of Fd^* on LK and LK on PSII. Together with the aforementioned positive dependence of the Calvin cycle flow on Fd^* , this circuit with positive feedback has the potential of supporting two steady states, one with a low and one with a high value of Fd^* (and correspondingly a low and high linear electron flow) depending on the input light intensity. (The states may also be selected by the availability of CO_2 and/or ATP,

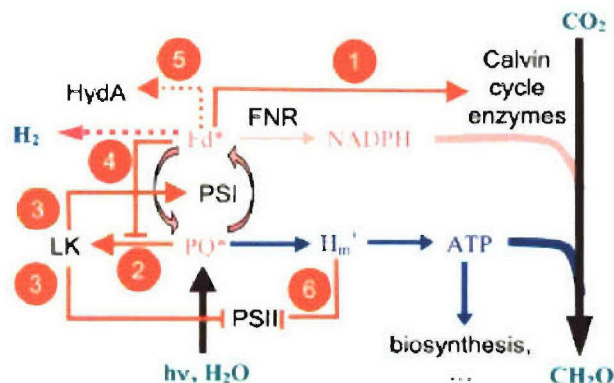


Figure 17: The photosynthetic reactions are represented diagrammatically along with some of the known regulatory links. The purple symbols indicate electron flow, blue symbols indicate proton flow, red symbols the regulatory links, and the dashed lines indicate the proposed reaction/link for hydrogen evolution. The regulatory links are: 1. Positive regulation of the activity and expression of Calvin cycle enzymes by reduced ferredoxin and other agents it reduces (the ferredoxin-thioredoxin reductase and thioredoxin). 2. LHCII kinase (LK) is activated by plastoquinol (PQ^*). 3. LK phosphorylates LHCII molecules normally in association with PSII; upon phosphorylation, LHCII moves laterally and associates with PSI, resulting in a net weakening of PSII and strengthening of PSI. 4. LK is deactivated by reduced thioredoxin regardless of its phosphorylation state. 5. Proposed activation of HydA expression by Fd^* or its associated reducing agents. 6. The negative regulation of PSII activity by a surplus of protons in the lumen.

since they determine the magnitude of the Calvin cycle flux.) If this positive feedback effect is indeed the driving force of the observed high-light/low-light behavior, then not much re-engineering of the upstream electron flux is needed for hydrogen evolution, since the latter amounts to replacing the native output module ($Fd^* \rightarrow NADPH \rightarrow \text{Calvin cycle flow}$) by $Fd^* \rightarrow H_2$ without affecting the core feedback loop. To mimic the control of redox potential on Calvin cycle enzymes, a regulatory control of the redox level on the expression of the hydrogenase (dashed red line of Figure 17) may be

added. In this way, the circuit will produce the desired behavior of expressing HydA and evolving hydrogen only under the condition of high light, without being affected by the CO₂ status.

Model 2 The situation can be very different if the feedback loop identified in Figure 17 is not the driving force of the two-state behavior. As an alternative scenario, we note that the Michaelis-Menton kinetics of catalysis by FNR produces a nonlinear sigmoidal dependence of the NADPH flux on Fd*, as it takes two Fd* molecules to reduce one NADP+ molecule to NADPH. The balance of the Fd*-dependent Calvin cycle flux with the NADPH flux then may in itself be sufficient to generate the two-state behavior. If this is the main cause of the bistable electron flow, then replacing this module by the one step reaction $\text{Fd}^* \rightarrow \text{H}_2$ will likely remove the bistability feature. Given how much of the chloroplast function depends on correctly discriminating the high-light/low-light environment, it may not be possible to significantly reduce the Calvin cycle flux by hydrogen evolution.

There are likely many other alternatives consistent with known facts; these must be sorted out for real progress in metabolic engineering to proceed.

8.3 Altering the ATP/pH-dependent Feedback on Photosynthesis

The Calvin cycle flux requires a balance of 2 NADPH and 3 ATP per CO₂ molecule. This is approximately the NADPH:ATP ratio provided by the linear electron flow. If the ATP flux available to the Calvin cycle is reduced due to demand by other cellular processes, the photosynthetic circuit can adjust the NADPH:ATP ratio to provide the additional ATP flux. This may be accomplished by the system by sending a portion of electrons from

the linear flow to the cyclic flow, presumably within the linear flow state without entering the state transition [12]. In the opposite situation, when there is a surplus of ATP flux, the system responds by reducing the activity of PSII; the NADPH:ATP ratio can be balanced by adjusting the conductance of ATPsynthase. The physiological function of this interesting response is believed to protect the photosynthetic reaction centers from the catastrophic consequences of photodamage, which may occur even upon brief exposure to intense radiation. This process is mediated by the increased proton level in the lumen, through the energy-dependent nonphotochemical quenching (qE) process, which harmlessly dissipates excessively absorbed light energy as heat. The innate photoprotection pathway may present a major challenge for hydrogen evolution, which if successfully implemented, would utilize only the electron flux and create a huge surplus of proton flux. Some of this proton flux can be converted to ATP flux demanded by cellular maintenance and biosynthesis. The remainder will need to be eliminated to reduce the proton level in the lumen and thereby to avoid the onset of photoprotection. Lee et al from ORNL propose to insert uncoupler proteins, such as UCP-1 and UCP-2, into the thylakoid membrane to reduce the proton level in the lumen. They propose to add a thylakoid targeting sequence upstream of the coding sequence of the uncoupler protein and place the recombinant gene under the control of the promoter of the hydrogenase gene. This is certainly a worthy effort to try, although possible post-transcriptional control mechanisms may hamper the expression of the recombinant construct. We suggest the use of the directed evolution method (above) to screen for variants of the uncoupler proteins (along with the target peptide and the UTR sequences) for those with superior performances.

Another issue of general concern regarding the uncoupler strategy involves the reduced viability of cells having reduced ATP synthesis activity,

even if the gene is placed under the control of a condition-specific promoter. It may be useful to put the expression of this gene under multiple control, so that it is, e.g., rapidly degraded when not expressed. More specifically from the metabolic pathway perspective, it is desirable to control the activity of the uncoupler protein to the ATP usage, so that the protein is only activated when the proton level in the lumen is the high. Otherwise, strong uncoupling activity may deprive the chloroplast of important maintenance activities that require ATP (e.g., replenishing the supply of the D1 protein in the reaction center), while weak uncoupling activity may not relieve the lumen of the undesirable proton accumulation. This will require detailed knowledge of the alga gene regulation mechanisms. Possibly, molecular evolution strategies may be employed to discover such regulatory pathways.

9 FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

We summarize this report with a list of findings, conclusions and recommendations:

9.1 General Findings Concerning Biofuel Production

1. Biofuels are advantageous because they inherently solve the storage problem posed by the diurnal fluctuation of sunlight. Additionally, they make carbon-carbon bonds, which are constituents of high value mobility fuels.
2. Biofuels are real, and not just a fantasy. A successful ethanol market exists in Brazil, and ethanol powers cars in Brazil. There is, however, a gap between what biofuels can currently do and what we need them to do to become a viable, material component to global energy demand (energy friendly, carbon neutral and economic.)
3. The science underlying biofuels is developing and much remains to be discovered and understood. There is likely room for significant improvement.
4. Photosynthesis has an upper bound on its solar energy conversion efficiency of 10%. The primary productivity for C4 plants in the field can be as high as 0.25%.
5. On the other hand, plants are not necessarily optimized to be energy conversion machines. For example, food crops have been genetically

improved to increase food production. and the efficiency of food production has not yet plateaued.

6. However, the reengineering of plants to improve biomass energy yield is a multi-axis problem. It is naive to think that a single (e.g., genetic) modification will solve the problem. Photosynthetic machinery has evolved to optimize fitness in a complex environment: Biological systems are intrinsically complicated because of the multiple feedback and control loops that must be present to guarantee robust survival. As a result, modifying any one property will likely have a limited leverage. It is important to think about the whole system (organism, environment, product, process).
7. Progress bridging these gaps requires a dedicated commitment to breeding and/or molecular and systems level analysis. These two approaches should be synergistic.

9.2 General Findings Concerning Fuel Production by Microorganisms

1. Microorganisms present a great opportunity for energy science, and hence are a natural focus for the Department of Energy. Microorganisms are simpler than plants; they have smaller genomes and proteomes and are easier to manipulate and culture.
2. The enormous biodiversity of microorganisms presents a broad palette of starting points for engineering. Microorganisms already make many metabolic products, some of which are useful fuels. It is likely that microorganisms will soon be synthesized ab initio.

3. The upper bound on the efficiency for oxygenetic photosynthesis in microorganisms is essentially the same as that for plants ($\sim 10\%$). Experimental measurements of efficiencies of fuel production must account for all system inputs and losses, including (but not limited to) pumping and sweeping out of products, stationary state relative to standard state, and the light intensity dependence of product yield.
4. Current microorganisms are not optimized for energy production of useful fuels. For example, hydrogen production from algae operates at less than 0.05% efficiency.

9.3 Recommendations and Conclusions

1. Boosting the efficiency of fuel formation from microorganisms is an important research challenge for the twenty first century. It is perhaps **the** major technological application for the emerging field of synthetic biology. In addition to the exciting opportunities for producing ethanol or hydrogen, microorganisms, either individually or in communities, might be used to directly produce liquid hydrocarbons. Realizing this potential requires both fundamental and applied research, and is a natural focus for the Department of Energy.
2. Engineering fuel production from microorganisms is a *systems* problem, requiring manipulation of multiple feedback and control loops. Fuel production processes (the dark reactions) are strongly coupled to the light reactions. Progress in both creating products and improving product yield requires recognition of the systems nature of this problem.

3. The systems biology of microorganisms is more tractable than that of plants, and thus microorganisms represent an excellent opportunity. The synergy between research into biofuel production by microorganisms and the Genomes to Life program is important and should be fully exploited.
4. Photosynthesis is an active and exciting area of current research, with major discoveries concerning the regulation and relative importance of components happening each year[6, 27]. These discoveries will play an important role in reengineering fuel production pathways in microorganisms.
5. The commonly quoted 10% upper bound in photosynthetic efficiency assumes that no energy is wasted in storing the photogenerated charges in chemical bonds. Additional losses will come from regulatory processes as well as maintenance energy expended to repair the components and insure system robustness. Until there is a systems level understanding of photosynthesis, it will be impossible to meaningfully more stringently bound the potential efficiency of photosynthetic fuel production.
6. Successful metabolic engineering requires a basic understanding of the system to be engineered. More understanding of photosynthetic regulation is necessary before metabolic engineering can reach its potential.
7. There is a pressing need for strategies to minimize the oxygen sensitivity of fuel-forming catalysts in biological systems. Hydrogenases, nitrogenases, and rubisco in C3 plants are all oxygen sensitive. Indeed, C4 plants are more efficient because they evolved an independent mechanism to isolate the rubisco from oxygen. Photodamage is a key

concern to any photosynthetic microorganism, and repair mechanisms have evolved to deal with this. Any new catalyst must be compatible with the existing repair machinery, or that repair machinery must also be redesigned. Directed evolution might prove to be a particularly promising strategy for improving these properties.

8. There is some opportunity to reengineer the photosynthetic components themselves to yield even higher energy conversion efficiency to the primary charge-separated products. This is a grand challenge, because of the interconnectivity and feedback loops already mentioned.
9. For carbon-based fuel production, a significant improvement in photosynthetic efficiency could be bounded by CO_2 supply constraints.
10. Even with an optimistic assessment of the potential for improvements, photosynthetic efficiency will lag behind that of man-made technologies (e.g., photovoltaic solar cells). For engineered microorganisms to succeed in the marketplace, their systems costs need to be significantly lower; however we are not aware of any systems-based cost analysis for solar H_2 generation from microorganisms. Such an analysis is needed to definitively understand the likely viability of this technology.

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